

2015

# Biological validation of ELISA results for the detection of Cry proteins

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**Biological validation of ELISA results for the detection of Cry proteins**

by

**Vurtice Carroll Albright III**

A dissertation submitted to the graduate faculty  
in partial fulfillment of the requirements of the degree of  
DOCTOR OF PHILOSOPHY

Major: Toxicology

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2015

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## **ABSTRACT**

The widespread use of Cry proteins in insecticide formulations and transgenic crops for insect control has led to an increased interest in the environmental fate of these proteins. Several detection methods are available to monitor the fate of Cry proteins in the environment, but enzyme-linked immunosorbent assays (ELISAs) have emerged as the preferred detection method, because they are cost-effective, easy to use, and provide rapid results. Validation methodology, which is essential to ensure accurate measurement of Cry proteins in environmental matrices, has been researched extensively, but most of this research has overlooked biological validation of ELISA results. This oversight has led to concerns that environmental studies utilizing ELISAs may be overestimating the concentrations of Cry proteins in the environment, which may affect the risk assessments for these proteins. A literature review discusses the history and usage of Cry proteins for insect pest control and discusses the use and validation of ELISAs for detection of Cry proteins in environmental samples. The concept of biologically validating ELISA results is introduced, and a critical review of published literature examines the state of ELISA usage and validation, including identifying areas for improvement. Eight different types of model systems were screened for their ability to produce fragments of Cry1Ab protein, and five of these model systems prove capable of generating Cry1Ab fragments. The fragments from these five model systems are then analyzed with ELISAs and bioassays to determine if the fragments are detectable or retain bioactivity. Fragments from four of the model systems are not detectable by ELISA and do not retain bioactivity. Fragments from the fifth model system are detectable by ELISA and do retain bioactivity. These results indicate that the use of ELISAs in environmental fate studies are providing an

accurate determination of the concentrations of Cry proteins in the environment and are not overestimating the concentrations. However, further work is needed utilizing additional model systems, including microbe-based model systems, in order to fully understand the fate of Cry proteins in the environment.

# **CHAPTER 1. A REVIEW OF CRY PROTEIN DETECTION WITH ELISAS**

*A paper to be submitted to the Journal of Agricultural and Food Chemistry*

Vurtice C. Albright III, Richard L. Hellmich, Joel R. Coats

## **Abstract**

The widespread use of Cry proteins in insecticide formulations and transgenic crops for insect control has led to an increased interest in the environmental fate of these proteins. Although several detection methods are available to monitor the fate of Cry proteins in the environment, enzyme-linked immunosorbent assays (ELISAs) have emerged as the preferred detection method, due to their cost-effectiveness, ease of use, and rapid results. Validation of ELISAs is necessary to ensure accurate measurements of Cry protein concentrations in the environment. Validation methodology has been extensively researched and published for the areas of sensitivity, specificity, accuracy, and precision; however, biological validation of ELISA results has been studied to a lesser extent. This review discusses the history of Cry proteins, their usage for insect control, the use and validation of ELISAs, and it introduces biological validation. The state of Cry protein environmental fate research is considered through a critical review of published literature to identify areas where the use of validation protocols can be improved.

## Introduction to Cry proteins

### *Origin*

Insecticidal crystalline proteins were first discovered, unknowingly, in 1901 when S. Ishiwata isolated a microorganism from a diseased *Bombxy mori* silkworm larva, which he named *Bacillus sotto* (1). [Although initially discovered and named by Ishiwata, because he did not formally describe it, Ernst Berliner received credit for naming it *Bacillus thuringiensis* when he discovered a similar microorganism in diseased Mediterranean flour moth larvae (*Anagasta kuehniella*) living in stored grain near the city of Thuringia, Germany in 1911 (2)]. In a follow-up report in 1905 Ishiwata noted “death occurs before the multiplication of the bacillus...” where the first indication that a toxin is at least partially responsible for the pathogenicity of *B. thuringiensis* arises (2-3). Another report in Japan provided more evidence that a toxin and not the bacterium itself was likely the responsible agent (2). However, identification of the toxic agent had to wait until 1954 when T.A. Angus showed that bipyramidal crystals present in sporulating *B. thuringiensis* cells were actually responsible for *B. thuringiensis* toxicity (2). He observed that the spores alone had no effect on *Bombxy mori* larvae, while dissolved crystals alone had the same effect as the spore-crystal complex and that as crystal count increased, toxicity increased as well (2,4).

From the 1960's to the 1980's, new *B. thuringiensis* subspecies such as *kurstaki*, *kurstaki HD-1*, and *tenebrionis*, were identified, bringing with them new crystalline proteins or Cry proteins (2). As more Cry proteins were discovered, a naming and classification system was needed to help identify the new proteins. The first attempt at classifying these toxins was proposed by Hofte and Whiteley (5). They originally



separated the Cry proteins into four major classes based on the host range and structure of the protein. Cry I proteins were lepidopteran-specific and were 130-140 kilodaltons (kDa) in size with a bipyramidal structure. Cry II proteins were significantly smaller, 65-70 kDa, and were cuboidal in structures. These proteins were also lepidopteran-specific and exhibited activity against dipteran species as well. Cry III proteins were coleopteran-specific proteins that also were smaller and had a rhomboidal structure. The last major class were the Cry IV proteins which were much larger (128-135 kDa), and had an ovoid structure. This class of Cry proteins was dipteran-specific only (5).

While this system was useful, it did have some inconsistencies that resulted in exceptions to the naming system. For example, in the Hofte and Whiteley nomenclature, Cry I proteins were lepidopteran-specific only; however, CryIC was reported also to be toxic to Dipterans (6-7). This is just one example, though several other exist. As a result of these inconsistencies, Crickmore *et al.* (7) developed a revised nomenclature system for the Cry proteins. Instead of focusing on target species and protein structure, the revised nomenclature groups brought together Cry protein toxins based on their sequence homology (7). Another major change is that the revised nomenclature switched from using Roman numeral to Arabic numerals in the primary rank (*i.e.*, Cry1Ab instead of CryIAb) to allow for newly discovered proteins to be integrated more easily. The revised nomenclature also established a committee to assist in naming newly discovered Cry proteins, whereas the prior system lacked the ability to maintain standardization (7). The committee also periodically reviews literature and publishes a comprehensive list of known Cry proteins, which is available at

[www.lifesci.sussex.ac.uk/home/Neil\\_Crickmore/Bt/intro.html](http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/intro.html). Since its introduction, the

revised nomenclature has been widely adopted. Currently, there are more than 70 primary classes of Cry proteins included on the comprehensive list.

### *Mechanism of Action*

The exact mechanism of action of Cry protein toxins is not currently known, but it is generally recognized as a multi-step process. Controversy over the final step or steps has led to the rise of three different models. The initial steps, however, are similar for all three models. First, the Cry proteins must be ingested by a susceptible species as they have no contact toxicity (8). Once inside the insect midgut, the Cry proteins are solubilized in an alkaline (for lepidopteran and dipteran insects) or a neutral/acidic (for coleopteran insects) environment (8). Proteases from the host insect, such as trypsin and chymotrypsin, then process the Cry proteins, cleaving off portions of the N- and C-terminals, leaving only the activated toxin (9). The activated toxin binds to specific receptors on the midgut epithelium (8). Here is where the three models diverge.

In the pore-formation model, the proteins bind to primary receptors in the insect midgut, such as cadherin-like receptors (10-11). The protein is cleaved to release the helix  $\alpha$ -1 of Domain I (11). Cleavage results in the oligomerization of the protein into a tetrameric prepore, which binds to secondary receptors such as aminopeptidase A (APN) and alkaline phosphatase (ALP) (10-11). The secondary receptors assist insertion of the prepore into the membrane, creating a pore in the membrane (10). Multiple pores in the midgut epithelial membrane results in a loss of membrane potential and the osmotic gradient as ions equilibrate across the membrane and water rushes into the cell (10). This influx of water results in cell lysis. Lysis of multiple cells compromises the integrity of the midgut and results in the insect's death (11).

In the signal-transduction model, the Cry proteins bind to the same primary receptor, cadherin, but instead of further cleavage and oligomerization, the binding initiates a  $Mg^{+2}$ - dependent signaling pathway through the activation of a guanine nucleotide-binding protein (G-protein) (10-11). The G-protein activation then triggers adenylyl cyclase which increases cyclic AMP (cAMP) levels and results in the activation of protein kinase A, leading to cytoskeleton and ion channel destabilization and the eventual death of the insect (10-11).

A third model has been proposed that combines elements of the previous two models. This model, known as the Jurat-Fuentes model after one of the authors who proposed it, suggests that toxicity of Cry1Ac to *Heliothis virescens* is the result of osmotic lysis and cellular signaling combined. In this model the Cry proteins bind to the cadherin-like protein HevCaLP, resulting in the activation of an intracellular signaling pathway. At the same time, the bound protein oligomerizes and binds to the secondary receptors APN and ALP, leading to pore formation. Both pathways then contribute to the death of the insect (10,12).

#### *Usage as Insecticides and Development of Transgenic Traits*

Insecticidal formulations containing a mixture of *Bacillus thuringiensis* spores and Cry proteins have been used to control pest insects for decades. Although the first commercial insecticide product was introduced in France in 1938 to target lepidopteran pests, it was not until the introduction of Thuricide<sup>TM</sup> in the late 1950's that these formulations were used on a wide scale (13-14). The use of Cry protein formulations increased through the 1960's and 1970's with the introduction of insecticides targeting coleopteran and dipteran species, eventually reaching \$100 million in annual sales in the

early 1990 (13). Sprays, powders, and other formulations of Cry proteins or the bacterium *B. thuringiensis* continue to be used in agricultural production on crops such as cotton, potatoes, tomatoes, and maize to control for several important coleopteran and lepidopteran pest species (15-16). Additional agricultural uses include sprays on fruit trees to control for light brown apple moth (*Epiphyas postvittana*) and on other crops like avocados, kiwi fruit, and strawberries (16-17). Cry protein-based insecticides also have found uses outside of agriculture. Aerial sprays are commonly used in forestry settings to control many pest insects including: gypsy moths (*Lymantria dispar* L) and the spruce budworm (*Choristoneura fumiferana*) in the United States; the pine processionary moth in Europe (*Thaumetopeea wilkinsoni*) and the Middle East (*T. pityocampa*); the nun moth (*L. monacha*) in Europe; the pine looper (*Bupalus piniaria*) in Sweden; the white spotted tussock moth (*Orgyia thyellina*) in New Zealand (18-20). Cry protein products are also under development to control plant-parasitic nematodes and termites (21-23). Control of both of these pests with *B. thuringiensis*-based formulations faces significant hurdles as survival of the bacterium (that germinates from spores in the formulations) in soil is poor (22).

In addition to plant protection, Cry protein-based insecticides also can be used in protection of humans. Many disease-causing organisms such as *Onchocerca volvulus* (river blindness) and *Wuchereria bancrofti* (lymphatic filariasis) can be vectored by black flies and mosquitoes (24-25). Control of the dipteran vectors is one key element in preventing the spread of these diseases. Since their introduction into the Onchocerciasis Control Program in West Africa in 1983 to help control the black fly, *Simulium damnosum*, Cry protein-based insecticides, along with conventional chemical

insecticides, have helped to significantly decrease the number of new infections (24-25). Formulations containing Cry proteins also are used to control black flies in Brazil (*Simulium pertinax*) and Argentina (*S. bonaerense*, *S. wolffhuegoli*, and *S. nigristrigatum*) on a purely nuisance basis, since no cases of disease transmission have been reported for these species (26-27).

Several formulation types of Cry protein-based insecticides have been developed to control mosquitos as they are vectors for some of the most significant human disease, such as malaria, dengue, and lymphatic filariasis. Sprays, dissolvable tablets, granules, and other Cry protein-containing formulations have been used throughout Asia and South America and have significantly reduced the occurrence of many of these human diseases (24-25). Additional formulations have been used in more temperate climates like Germany and the United States to control for other diseases, such as West Nile virus, and as nuisance control (24-25).

Currently, Bt formulations are primarily used by gardeners, organic farmers, and in forestry (14). Their widespread use in agriculture has been hindered by several factors. Cry protein formulations are not stable in the environment, resulting in low residual activity (14). This lack of persistence triggers the need for multiple applications at 5-7 day intervals to ensure protection is achieved; such near constant spraying is not economically viable as the costs of fuel, labor, and insecticides can accumulate rapidly (28). The narrow specificity of Cry protein formulations, while beneficial for protecting non-target organisms, can lead to the application of multiple formulations to manage multiple pest species. Formulations containing Cry proteins typically lack systemic activity and cannot penetrate plant tissues to reach pest insects within a plant, such as

European corn borers (14). Finally, another factor that has helped curtail the use of Cry protein formulations in agriculture has been the development and rapid increase in the use of transgenic plants producing Cry proteins for protection against pest insects.

Development of transgenic plants expressing genes for the production of Cry proteins occurred near simultaneously in multiple laboratories. Fischhoff *et al.* (29) developed transgenic tomato plants for the control of several lepidopteran pests using both the full length coding sequence from *Bacillus thuringiensis* var. *kurstaki* HD-1 (*B.t.k.* HD-1) and a coding sequence for truncated versions. Three plants transformed with the full length gene were produced, while only two plants with the truncated gene were created. However, both plants with the truncated version of the gene achieved 100% mortality of *Manduca sexta* larvae, while only one of the three plants with the full length gene had insecticidal activity, and at much lower levels (50-80% mortality). The authors then focused solely on the truncated genes and were able to develop plants capable of achieving 100% mortality of larvae in 48-72 hours (29). Meanwhile, other labs were developing transgenic tobacco plants also to control for *M. sexta*. Vaeck *et al.* (30) also attempted to transform both a full length *Bt* gene isolated from *B. thuringiensis* strain *Berliner 1715* and several truncated versions of the gene into tobacco plants. They were successful in these transformations; however, when compared, they observed no insecticidal activity in any of the plants transformed with the full length gene. The truncated versions performed significantly better with 100% mortality observed after six days in plants transformed with the truncated NPT860 gene. F<sub>1</sub> progeny of two of the transformed plants also were tested for insecticidal activity and achieved 100% larval mortality after five days, thus showing that the *Bt* genes in transformed plants are

inheritable (30). Barton *et al.* (31) also were developing transgenic tobacco plants for protection against *M. sexta*, but with a different trait. Initial efforts focused on inserting a full length gene from *B.t.k.* HD-1 into tobacco plants. However, these plants did not prove viable, and their efforts were quickly shifted to focusing on only the truncated version of the gene. Not long after, they had developed transformed plants with truncated genes that were capable of achieving mortality at levels similar to those observed in the previous two studies (31).

While many transgenic plants performed well in the efficacy tests, not many produced protein at high levels in the plant tissues. This led to concerns that these plants might not be commercially viable (32). Thus, several modifications to the gene structure were performed in attempts to increase the amount of insecticidal Cry protein produced in the plant tissues. Changing promoters, leader sequences, and fusion proteins did not lead to significant changes in protein production (30-31). This led Perlak *et al.* (32) to focus on modifying the DNA sequence of the *Bt* genes in tomato and tobacco plants. Partial modifications (removal of sequences predicted to inhibit gene expression) and full modifications (removal of ATTTA sequences and potential polyadenylation sequences, using plant preferred codons, etc.) were performed. These modifications produced substantial results; some plants containing the partially modified gene had 10-fold higher protein levels and some plants with the fully modified gene had 100-fold higher protein levels (32). Other modifications were able to increase the amount of protein in cotton from less than 0.002% of the total protein to 0.05-0.1% of the total protein (33). McBride *et al.* (34) also recognized the need for increasing protein levels but decided that producing synthetic genes was too expensive and labor intensive. They chose to focus on

expressing the gene in chloroplasts, instead of the nuclear DNA because the plastid's genome is AT-rich, much like *Bt* genes and unlike plant genes which are typically GC-rich. This transformation resulted in significant improvement in Cry protein production in the plant. As much as 5% of the total soluble protein was Cry protein using this method (34).

Following up on these successes, other transgenic crops were quickly developed, including cotton to control for cotton bollworm (*Heliothis zea*), rice to control for striped stemborer (*Chilo suppressalis*) and leafroller (*Cnaphalocrosis medinalis*), and potato to control for Colorado potato beetle (*Leptinotarsa decemlineata*) (33,35-36). Field trials were performed to determine the efficacy of transgenic plants to control pest insects under more natural conditions. Tomato plants artificially infested with *M. sexta* exhibited only limited feeding immediately after hatching near the site of introduction, while the non-transgenic controls were entirely defoliated after two weeks (37). The transgenic tomato plants also reduced damage caused by *H. zea* (natural and artificial infestations) and *Keiferia lycopersicella* (natural infestations); however, significant damage still occurred in the tomato fruits, possibly as a result of insufficient levels of the Cry protein in the fruit (37). Six different maize plant varieties expressing Cry1Ab protein were field tested against two generations of artificial infestations of European corn borer (*Ostrinia nubilalis* Hübner). Despite the heavy infestation pressure, excellent protection was reported. Foliar damage to transgenic maize rated an average of 3 on a modified Guthrie scale (1 – no visible injury, 9 – severe leaf injury) while control plants had an average rating of 6.8 (38). Additionally, mean tunnel length in transgenic maize ranged from 1.7 –



7.2 cm while in non-transgenic control plants the mean tunnel length averaged 28 – 144 cm (38).

All of this research led to the approval in 1995 of the first transgenic crops conferring insect resistance for commercial use (39). Included in the first round of approvals was Maximizer<sup>TM</sup> maize (Ciba-Geigy), Bollgard<sup>TM</sup> cotton and New Leaf<sup>TM</sup> potatoes (both Monsanto products) all of which were first planted commercially in 1996 (39-40). The next year saw additional approvals of insect-resistant maize products from Monsanto and Northrup King. Initially, only traits for the control of corn borer (maize), Colorado potato beetle (potato) and tobacco budworm, bollworm, and pink bollworm (cotton) were available. Traits for control of corn rootworm and corn earworm in maize were introduced commercially in 2003 and 2010, respectively (40). Since these initial introductions, transgenic cotton and maize expressing Cry proteins for insect resistance have been widely adopted. As of 2015, 81% of maize and 84% of cotton planted in the United States have expressed one or more insecticidal traits for protection against a variety of insect pests (41). As previously mentioned, New Leaf<sup>TM</sup> potatoes were introduced at the same time as the first transgenic maize and cotton plants. However, while transgenic maize and cotton use increased rapidly, New Leaf<sup>TM</sup> potato usage faltered as sales never rose above 5% of the total amount of potato seed sold and it was eventually pulled from the market after only six years (42).

Although maize and cotton are the only transgenic crops expressing insecticidal traits currently in use, other crops are in various stages of development. Development of transgenic tomato, tobacco, and rice plants expressing genes for production of insecticidal Cry proteins has already been mentioned in this review (29-32,34-35,37).

However, these research efforts have yet to produce any commercially available products (40,43). In addition to the aforementioned crops, other crops have been developed to produce Cry proteins for insect resistance, and some have even been approved for commercial usage in other countries. Transgenic soybean plants have been engineered to produce Cry1Ac for the control of *Helicoverpa zea*, *Helioverpa armigera*, *Pseudoplusia includes*, *Heliothis virescens*, *Anticarsia gemmatalis*, and *Hypena scabra* (44-46). This trait, however, exhibited little or no effectiveness against *Spodoptera litura*, *S. exigua*, and *Agrotis ypsilon* (46). Though not commercially available in the United States, a transgenic soybean plant producing the Cry1Ab protein was registered for commercial use in Brazil in 2011 (47).

Several other vegetable species also have been genetically modified to produce Cry proteins for insect protection. The most successful, and only one currently in commercial usage, is sweet corn engineered to produce Cry1Ab. It was first introduced in 1998 by Novartis Seeds, and initially struggled to gain a large market share due to a lack of public acceptance; however, it is currently estimated that Bt sweet corn accounts for 18-25% of the total fresh sweet corn market (data on usage in processed sweet corn not available)(48-49). Another transgenic plant close to commercial usage is the brinjal, or eggplant. A transgenic brinjal producing Cry1Ac for control of the fruit and shoot borer (*Leucinodes orbonalis*) was approved for commercialization in India in 2009, but never reached market due to significant political pressure (50). However, it was released to a limited number of farmers in Bangladesh in early 2014 (51). Numerous *Brassica* species producing various Cry proteins also have been developed to control for the diamondback moth, *Plutella xylostella*. Transgenic cabbage producing Cry1Ab and Cry1B, transgenic

broccoli producing both Cry1C alone and in a pyramid with Cry1Ac, transgenic cauliflower producing Cry1C or Cry 9Aa, and transgenic canola producing Cry1Ac all have been shown to have excellent control against diamondback moth larvae (52-57). Additionally, activity against the cabbage looper, *Trichoplusia ni* was observed for the Cry1C cauliflower and the Cry1Ac canola (52-53). Cry1A-producing rutabaga has been developed for control of the cabbage caterpillar, *Pieris rapae* (58). Despite the extensive research into these transgenic *Brassica* vegetables, viable commercial products have not yet been developed. In an early effort to commercialize some of these products, a private-public partnership was formed in 2003 with the intent of making pyramided Bt cabbage and cauliflower available for commercial use in India; however, the collaboration ended in 2010 when a major partner backed out before any commercial products could be brought to market (48).

Two non-traditional crop plants under development that produce insecticidal Cry proteins are poplar and eucalyptus trees. Poplar trees are important to the paper and timber industries due to their rapid growth rate, but are susceptible to damage from a variety of coleopteran and lepidopteran pests, including *Chrysomela tremulae* and the gypsy moth *Lymantria dispar* (59-60). Transgenic poplar trees have been developed to provide protection against either coleopteran or lepidopteran pests using the *cry3Aa* gene and a modified *cry1A* gene respectively (59-60). Significant protection is incurred by these traits with 67-89% mortality of *L. dispar* larvae and 100% mortality of *C. tremulae* larvae and adults (59-60). The other non-traditional transgenic plant expressing a Bt gene currently under development is a eucalyptus tree expressing the *cry3A* gene (61). Like poplar trees, eucalyptus trees are important sources for timber and pulp for paper,

primarily in Australia, and are susceptible to defoliation by the Tasmanian eucalyptus leaf beetle (*Chrysophthara bimaculata*) (61). Eucalyptus trees expressing the *cry3A* gene have been shown to have excellent protection against *C. bimaculata* with 87% larval mortality after only four days (61).

#### *Non-Target Toxicity Concerns*

Although individual Cry proteins have a narrow spectrum of activity against specific insect orders, some non-target effects have been reported. Much work has focused on monarch butterflies (*Danaus plexippus*) after a paper by Losey *et al.* (62) reported decreased growth rates and survival of monarchs fed with pollen from transgenic Bt corn in a laboratory study and Jesse and Obrycki (63) reported lethal effects from a field study. Follow-up studies determined only one type of Bt maize (Event 176), which is no longer commercially available, was lethal to monarch larvae (64-66). Effects from other types of commercially available maize were expected to be minimal due to low toxicity and low exposure (67). Continuous exposure of larvae to pollen from Cry1Ab Bt maize throughout development, a worse-case exposure scenario, found evidence of reduced feeding, decreased weight gain, longer development time, and increased larval mortality (66). The estimated additional mortality to monarch population due to this exposure was only 0.6% (66).

Other non-target insects in the order Lepidoptera also have been reported to be susceptible to Cry1Ab. *Spodoptera littoralis* exposed to Cry1Ab were reported to have decreased larval survival and weight gain and increased development time in laboratory studies (68). The lycaenid butterfly *Pseudozizeeria maha* fed on Cry1Ab-containing pollen in laboratory studies exhibited decreased larval survival (69). Higher mortalities

and decreased weight gain also have been reported for *Pieris brassicae*, *Pieris rapae*, and *Plutella xylostella* in laboratory studies (70).

Some closely related Trichopteran species have been reported to be susceptible to lepidopteran-active Cry proteins. Decreased growth rates have been reported for the caddis fly *Lepidostoma liba* fed transgenic maize detritus; the detritus was not analyzed, so the quantity and quality of the Cry proteins the larvae were exposed to is unknown (71-72). (Although subsequent studies refute this claim, see 73). Increased mortality and decreased abundance in fields where Cry1Ab was present have been reported for *Helicopsyche borealis* and *Pycnopsyche* sp. respectively (71,74). Effects on non-target Dipterans have been reported with the aquatic midge *Chironmus dilutus* showing decreased survival on Cry3Bb1 and the crane fly *Tipula abdominalis* exhibiting decreased growth rates on transgenic Cry1Ab maize (though the latter may be due to tissue differences between transgenic maize and near-isoline non-transgenic maize) (73,75). A non-insect species, the isopod *Caecidotia communis*, also was reported to have decreased growth rates on Cry1Ab maize (though again, tissue differences may account for the differences)(73). Another study reported that Cry1Ab had no effects on two isopod species; however, difference in nutritional content between corn hybrids were likely responsible for differences in *Trachelipus rathkii* growth (76). [For further reading on the effects of Cry proteins to non-target organisms, see 77-79].

#### *Environmental Chemistry*

As a result of these potential non-target toxicity issues, it is important to address questions surrounding the environmental fate of Cry proteins. Environmental fate questions that must be addressed include, but are not limited to: degradation, persistence,

mobility, and bioavailability of the Cry proteins. However, before any of these questions can be answered, researchers must have adequate tools for detecting and quantifying the amount of Cry proteins in the environment. There are currently a wide variety of detection methods available for Cry proteins, including high-performance liquid chromatography/mass spectrometry, biological assays (bioassays), and Western blotting. A drawback of these methods is that they are time- and labor-intensive and as a result, are generally cost-prohibitive for most researchers. Thus, most researchers elect to use enzyme-linked immunosorbent assays (ELISAs) to detect Cry proteins in environmental matrices.

## **Introduction to ELISAs**

### *Theory*

Immunoassays harness the power of the adaptive immune system in which lymphocytes produce antibodies in response to foreign molecules such as bacteria and viruses (80). These antibodies have high specificity to epitopes on the foreign molecule they target. If the binding of an antibody to a foreign molecule can be visualized, then antibodies could be used for the detection of specific foreign molecules (81). Normally, one end of the antibody binds to the antigen while the opposite end binds to a phagocyte, which will ingest and destroy the invading molecule (80). However, in an immunoassay, the phagocyte-binding end of the antibody is conjugated to a reporter molecule. Early immunoassays used radioactive labels as the reporter molecule attached to the antibodies. However, due to concerns regarding worker safety and handling and disposal of radioactive materials, alternative reporter molecules were sought, including using enzyme-labeled antibodies (82). The first paper reporting the use of enzyme-labeled

antibodies was published in 1971 by Engvall and Perlmann. The assay developed in that paper, which the authors called an enzyme-linked immunosorbent assay or ELISA, conjugated an enzyme, instead of a radiolabeled molecule, to an antibody to quantitatively measure immunoglobulin G in rabbit serum (82-83). Since the publication of that paper, ELISA use has increased dramatically across many scientific disciplines.

ELISAs are the most popular method currently used to detect Cry proteins in environmental samples. There are several types of ELISA methods currently available including direct, indirect, and competition ELISAs; however, sandwich ELISAs are the most commonly used type for detection of Cry proteins (84). In a direct sandwich ELISA, a capture antibody is bound to a solid phase, such as the bottom of a well in a 96-well plate (Figure 1). The sample containing the antigen (in this case, the antigen is the Cry protein of interest) is then added to the well, and the antigen binds to the capture antibody. The wells are then washed, removing any unbound antigen. The detecting antibody conjugated to an enzyme is added and binds to a different epitope on the antigen, forming a protein sandwich. After another wash step, a substrate is added which is processed by the enzyme, producing a colorimetric response indicating the presence or absence of the antigen. ELISAs for detection of Cry proteins can be made quantitative through the use of a standard curve to provide a general idea of the amount of a specific Bt protein present (81,84-85). Indirect sandwich ELISAs also may be used instead of direct sandwich ELISAs. Indirect sandwich ELISAs are similar to direct sandwich ELISAs, except that they use a second capture antibody that is not conjugated to an enzyme to bind to a different epitope on the antigen. The detecting antibody then binds to the second capture antibody (81,85).

### *Antibodies*

Foreign molecules, or antigens, can be injected into a mammalian host (i.e., mice, rabbits, etc.). The presence of the antigen then elicits the immune system of the host animal to produce antibodies specific to the antigen of interest (81). By using a Cry protein as the antigen being injected, antibodies specific to that protein can be produced. The two main types of antibodies currently used in ELISAs are polyclonal and monoclonal antibodies. Polyclonal antibodies are produced by injecting a host organism with the antigen of interest and collecting and purifying the host's serum. This results in a heterogeneous mixture of numerous antibodies with varying specificities and affinities for the antigen of interest. The heterogeneous mixture can lead to very sensitive immunoassays, as having multiple antibodies present increases the chances of antigen recognition and binding. However, this can also lead to non-specific binding to other antigens with similar epitopes. Polyclonal antibodies are the easiest and cheapest to produce in large quantities, but their availability is limited to the life span of the host. Additionally, it is not possible to reproduce the exact serum from another host due to variability between individuals of the same species (81,86-87).

Monoclonal antibodies consist of a homogenous mixture of a single antibody. They are produced by injecting a host organism with the antigen of interest and then collecting and fusing individual lymphocytes from the host to myeloma cells, resulting in a hybridomal cell line that can produce a single type of antibody. Multiple hybridomas are screened to identify those hybridomas that produce the most antibodies with the best binding ability. Though they are much more time-consuming and expensive to set up, once established, the hybridoma can produce the specific antibody for extended periods



of time in tissue culture. Non-specific binding is less of an issue with monoclonal antibodies, however, sensitivity can be lowered if the targeted epitope is damaged or altered significantly (81,86-87).

#### *Current usage*

One of the first studies to investigate the use of ELISAs for detecting Cry proteins was Wie *et al.* (88). The authors investigated the prospect of using ELISAs to detect and quantify the amount of crystal toxins from *B. thuringiensis* subspecies. They determined that the ELISA method was highly accurate and extremely sensitive. While specificity between dipteran-active and lepidopteran-active toxins could be achieved, there was significant cross-reactivity between lepidopteran subspecies of *B. thuringiensis* (88). Since that initial study, however, significant amounts of research have been devoted to improving the antibodies used and the ELISA procedure as a whole (86,89-92). Early use of ELISAs for detection of Cry proteins required developing antibodies in the laboratory for the specific protein of interest (93). More recently, commercial manufacturers have been developing ready-made kits with antibodies specific to 1-2 Cry proteins. Table 1 contains a list of ready-made ELISA kits available from several manufacturers.

There are a wide range of uses for ELISAs currently in agriculture. ELISAs can be used to screen cell cultures or plants for the presence of a novel protein of interest to determine which cell culture or plant is expressing the protein after genetic transformation in a laboratory. These cell cultures or plants can then be further screened to remove cultures or plants that are expressing the protein at insufficient levels. As trials expand into greenhouses or field settings, ELISAs can be utilized in event selection to identify low trait expressing plants, allowing them to be culled from the gene pool (94).

Once a product is ready for commercial usage, ELISAs can be used to determine protein expression levels in roots, leaves, pollen, and other various plant tissues. Such information is typically required by regulatory agencies as part of the extensive registration packages. ELISAs also may be employed as quality assurance/quality control tools during seed production to ensure the products (i.e., seeds) being delivered to the customers will perform as promised (95). Following harvest, ELISAs can be used to identify transgenic crops from non-transgenic crops and ensure that transgenic crops do not become mixed with non-transgenic crops throughout the supply and processing chain, where required by governmental regulations (96).

One of the biggest use of ELISAs in agriculture research, and the focus of the remainder of this paper, is for monitoring of Cry proteins in environmental matrices pre- and post-harvest. As with any pesticide, insecticides containing Cry proteins may enter soil and water matrices through direct application, such as foliar sprays and soil drenches, or indirect application, such as spray drift and spills. Cry proteins also may move into water via surface water runoff and soil erosion. Another pathway that Cry proteins in transgenic crops can enter the ecosystem is through the transportation of crop material. This typically occurs post-harvest as crop residues are incorporated into the soil or transported to water bodies (via wind or surface water runoff), and begin to degrade, releasing Cry proteins (71,97-98). Cry proteins also may enter soil and water bodies through exudation from plant roots or via pollen deposition (62,99). Understanding the movement and fate of Cry proteins in the environment is crucial in determining the risk of Cry proteins to non-target organisms.

Presence/absence, dissipation, persistence, and partitioning of Cry proteins in soil and water matrices can all be addressed by ELISAs for both insecticide formulations containing Cry proteins and transgenic crops producing Cry proteins. In lab studies, Douville *et al.* (100) spiked soil and water samples with pure Cry1Ab protein to determine persistence in the environment. Aquatic environments degraded the protein more rapidly than soils, with half-lives of 4 and 9 days respectively (100). This result was corroborated by other research groups. In a study on the aquatic fate of Cry3Bb1, half-lives of Cry3Bb1 protein in various maize tissues in aquatic microcosms were less than three days, and no Cry3Bb1 was detected in the water or sediment (75). A related study looked at dissipation of Cry3Bb1 in maize tissues in soils, and determined there was a slightly longer half-life for the various maize tissues in soil (101). Another research group showed that two different formulations containing Cry4, a mosquitocidal protein, were below the limit of detection of the ELISA after 7 days (LOD = 2 ppb) in aquatic microcosm studies (92).

In field studies, the information on persistence and dissipation is mixed. Sundaram *et al.* (102) investigated persistence of a commercial Bt *kurstaki* formulation Foray® 48B, on oak leaves following foliar application to protect against the gypsy moth *Lymantria dispar* L. Their results indicated that the amount of endotoxin present was below the limit of quantification of 8 ppb after only two days (102). Gruber *et al.* (103) studied the presence of Cry1Ab in soil in fields following cultivation of transgenic maize in the same four fields for nine consecutive years. The authors found no evidence for accumulation or persistence of Cry1Ab during long-term cultivation as only one site had protein levels above the limit of detection six weeks after harvest, and no protein was

detected at any of the sites the following spring (103). Daudu *et al.* (104) tracked the degradation of Cry1Ab in leaf and stem residues in litter bags buried in soils and found that after 14 days, less than 0.02% of the Cry1Ab remained in the leaf and stem residues. In addition, no Cry1Ab protein was detected in the soil around the litter bags, indicating that the Cry protein was rapidly degraded (104).

In contrast, Baumgarte and Tebbe (105) studied the amount of Cry1Ab protein in soil and plant tissue residues in two agricultural fields where transgenic maize was grown. Cry1Ab was detected in soils and plant tissues at both field sites during the growing season and post-harvest, with Cry1Ab still detected in one field seven months later (105). Tank *et al.* (106) found free Cry protein in 23% of 215 water samples taken from streams near agricultural fields six months after harvest.

#### *Advantages and Disadvantages*

The use of ELISAs for detection of Cry proteins in environmental matrices has several advantages and disadvantages. ELISAs are highly sensitive, with detection limits in the ng/mL range, and are highly selective due to the specificity of the antibodies (96,107). ELISAs are easier to perform and produce results more quickly than conventional methods like liquid chromatography paired with mass spectrometry (LC/MS). While the amount of time dedicated to sample preparation may be similar, analysis of the samples is much faster with ELISAs. For example, analysis of a 96-well microtiter plate designed to detect Cry1Ab or Cry1Ac protein requires two hour incubation with the sample and antibodies, a washing step, and 20 minute incubation with the substrate to allow for a colorimetric change to occur (108). If plating of the samples and read time on a plate reader are included, the total analytical time is 3.5-4 hours, or 2-

2.5 minutes per sample, while analysis with LC/MS could take 10-15 minutes per sample (including re-equilibration time), for a total of 16-24 hours. The ability to process a large amount of samples in a short time span also makes ELISAs cost effective (96). As of May 2015, a 480 well Cry1Ab/1Ac kit from Agdia, Inc. (Elkhart, IN, USA) cost approximately \$600 USD, or \$1.25 per sample (not including controls) (109). Depending on equipment set-up, the cost to operate a LC/MS can be \$30-100 per hour. Assuming costs are \$30 per hour, and run time per sample is 10 minutes, to analyze 480 samples with LC/MS would cost \$2,400 (\$5 per sample).

A drawback with the use of antibodies, however, is that the antibodies may cross-react with closely related proteins, such as antibodies specific for Cry1Ab cross-reacting with Cry1Ac (107,110-111). Also, non-specific binding may occur between other proteins in environmental samples and the antibodies, enzymes, or even the plastic microtiter plates utilized in the assays (80,112). Further, if the protein structure is altered or the antibody binding sites are damaged, there can be reduced antibody-antigen binding, which may affect the accuracy of the analysis (96). Conversely, ELISAs can only distinguish between bioactive and non-bioactive proteins when the protein structures are altered significantly. Thus, if only minor structural alterations exist that render the protein inactive against susceptible insect species, but do not affect the binding of the antibodies to the protein epitopes, then a protein that is not bioactive may be detected by the assay, producing a false-positive result (93,113). Finally, ELISAs are not readily conducive to multi-analyte analysis (110). Some attempts have been made to allow for detection of multiple analytes, but these are not true multi-analyte analyses, as they require dividing a microtiter plate into multiple sections, one for each analyte. This can

greatly reduce the number of samples per plate; if samples are screened for three different analytes, the number of samples per plate decreases from 96 to 32 (114).

## **Validation of ELISAs**

As with any analytical method, validation of ELISAs is necessary to ensure that the performance of the assay meets specific criteria. These criteria, as well as the scope of the validation procedure, may vary according to the intended use of the assay; however, at a minimum, validation procedures typically address the sensitivity, specificity, accuracy, and precision of ELISAs to ensure that the target analyte can be detected and/or quantified in a reproducible manner. Several excellent articles and book chapters have been published on the topic of ELISA validation and cover the topic in greater detail than in this review (81,84,112). Additionally, several articles have been published describing validated methods for detection of Cry proteins in various matrices (111,115-117). The remainder of this section will briefly cover the key areas of validation: sensitivity, specificity, accuracy, and precision.

### *Sensitivity*

The sensitivity of an ELISA is determined by the smallest amount of target analyte that an assay can reproducibly detect. For a quantitative ELISA, determining the quantitative range, upper limit of quantitation (ULOQ) and lower limit of quantitation (LLOQ) are important parameters for defining sensitivity. The quantitative range is the range over which the ELISA will produce quantitative results within acceptance criteria; it is determined by the concentrations over which the standard curve produces a linear response. The upper limit of quantitation and lower limit of quantitation are the highest and lowest concentrations, respectively, that can be measured with an acceptable level of

accuracy and precision. They are commonly defined as the highest and lowest points, respectively, on the standard curve. The sensitivity for a qualitative ELISA is often defined by the limit of detection, or the lowest concentration at which it is possible to differentiate between a positive and negative sample. The limit of detection can be defined as an absorbance reading that is two or three standard deviations above a negative or background control sample (84,112).

### *Specificity*

The specificity of an ELISA is the capacity of the assay to differentiate between the Cry protein of interest and other components that may be present in the samples. There are two main constituents to specificity: cross-reactivity or interference of other transgenic proteins, and cross-reactivity or interference of matrix components. Checking for cross-reactivity with other proteins (including transgenic proteins) is essential to determining if the antibodies utilized in the ELISA will bind to other closely related proteins, such as Cry1Ab and Cry1Ac. Fortunately, for researchers using commercial ELISA kits, this work is typically performed by the manufacturer. Equally important is determining if the matrix (i.e., soil, tissue, water) can affect the capacity to detect and/or quantify the Cry protein of interest. Matrix components may contain homologous endogenous counterparts that could potentially cross-react with the antibodies, producing a response. Non-specific binding between the antibodies and matrix components also may produce a positive response, while decreased ability of the assay to quantify the protein may occur if matrix components interfere with the antibody-Cry protein interaction (112).

### *Accuracy*

The accuracy of an ELISA is its ability to determine the true amount of Cry protein in a sample. Accuracy of an ELISA can be determined through the use of extraction efficiency and fortification-and-recovery studies. Extraction efficiency is used to express the capability of an extraction method to separate the Cry protein of interest from the sample matrix, which is determined by utilizing serial extractions of the same sample. The amount of protein in the first extraction is divided by the sum of the protein in all the extractions. Extraction efficiencies between 70-100% with a coefficient of variation (CV) of less than 20% are ideal. Fortification-and-recovery testing (also known as spike-and-recovery) is used to determine recovery across multiple points in the quantitative range. In this test, negative soil, tissue, or water samples (samples free of Cry proteins) are fortified or spiked with a known amount of protein. The samples are then extracted once according to the extraction procedure, and the amount of protein recovered is divided by the total amount of protein initially added. Ideal mean recovery values are between 70-120%, with a CV of less than 20% (84,112). The inherent variability in biological systems and the small quantities of Cry proteins that are typically found in environmental samples make accurate measurements challenging and can lead to recovery values greater or less than 100%. Some major factors in the extraction process that can affect the recovery of Cry proteins in the extraction efficiency and fortification-and-recovery procedures from various environmental matrices includes the type of extractant/solvent used, number of times a samples is extracted and the duration of each extraction, and the type of agitation used (grinding, shaking, etc.).



### *Precision*

The precision, or reproducibility, of an ELISA describes the amount of variation that may occur within an assay or across multiple assays. The use of commercial ELISA kits can help reduce some of this variability, especially if kits from the same lot number (i.e., same source of antibodies, enzyme conjugate, etc.) are used for all samples. However, not all sources of variability can be accounted for by the use of commercial kits, such as day-to-day and analyst-to-analyst variability, and thus, the precision of the assay needs to be verified. Assay precision can be tested by analyzing aliquots of the same quality control samples of known concentration multiple times (across days, analysts, etc.). The mean and standard deviation of all of these samples can then be used to calculate the coefficient of variation for the samples. Ideally, the coefficient of variation will be less than 20%, though this can vary depending on the intended use of the assay (81,84,112).

One way to think about accuracy and precision is to imagine a dart board (Figure 2). In this analogy, the center ring of the dart board, or bulls-eye, represents the true amount of Cry protein in a sample. Method validation ensures that an analytical method has all samples tightly grouped in the center ring; this method is then said to be both accurate and precise (reproducible) (Figure 2A). Failure to validate an analytical method may produce results that are reproducible, but inaccurate (Figure 2B), accurate, but not reproducible (Figure 2C), or neither accurate nor reproducible (Figure 2D) (81).

## Biological Validation

The procedures and criteria for validating the analytical performance of ELISAs are well described, as previously discussed. One area that is not well defined is the biological validation of ELISA results. False-positive ELISA results, a positive detection when no antigen is present, are a known issue with ELISAs across many areas of science (118-120). One potential source of false-positive results when analyzing environmental samples for Cry proteins is the detection of partially degraded proteins by the ELISA. This may result from the prevalent use of polyclonal antibodies in commercial ELISA kits. Polyclonal antibodies bind to multiple epitopes on a protein; therefore, a partially degraded protein may have a sufficient number of antibody binding sites still intact to allow for a positive detection.

Several published studies support this possibility. Einspanier *et al.* (121) collected samples from the gastrointestinal tract (GIT) at slaughter of cows fed either transgenic Cry1Ab maize or non-transgenic isoline maize; the samples were analyzed with a commercial ELISA kit for Cry1Ab/1Ac. The results indicated that Cry1Ab protein resisted digestion and appeared to accumulate in some intestinal juice samples. Cross-reactivity of the ELISA with animal, microbial, or plant compounds was ruled out because this phenomenon was not observed in cows fed non-transgenic isoline maize. In a follow-up study, the authors hypothesized that the positive ELISA detection may have been the result of a fragmented, yet immunoreactive Cry1Ap protein reacting with the antibodies (119). This hypothesis was tested in a second feeding study. Cows were fed either transgenic Cry1Ab maize or non-transgenic isoline maize and GIT samples were collected at slaughter. In addition to ELISAs, Western blotting was performed on all

samples. The ELISA results emulated the initial study; Cry1Ab protein was detected in all samples and the concentration appeared to increase during passage through the GIT. Cross-reactivity with animal, microbial, or plant components was again ruled out. The Western blot data told a different story. Fully intact Cry1Ab was not detected in any GIT samples; fragment bands at approximately 17 and 34 kDa were observed in cows fed transgenic maize, while no comparable protein bands were observed in cows fed non-transgenic maize. The results support the hypothesis that the Cry1Ab protein was fragmented, yet still capable of immunoreacting with the ELISA antibodies (119).

Similar results were observed in earthworm tissue samples. Emmerling *et al.* (122) collected samples of earthworm casts and gut content and analyzed the samples for the presence of Cry1Ab with ELISAs and Western blotting. ELISA results indicated a decreasing, yet still detectable concentration of protein as the protein moved through the earthworms' digestive tract. However, no fully intact protein was observed in any of the samples. Three fragments with an approximate size of 17, 23, and 31 kDa were detected in the foregut and midgut samples. Western blotting did not indicate that fragments were present in samples of the hindgut or cast material, even though Cry1Ab was detected in these samples by ELISA. The authors did not specify if blank control samples were performed; thus, positive ELISA detections in the hindgut and cast samples may be the result of cross-reactivity with animal, microbial, plant, or soil components (122).

These studies provide examples of why validation is necessary to ensure that only fully intact Cry proteins are being detected by ELISAs. Detection of non-bioactive Cry protein fragments by ELISAs may lead to an overestimation of the amount of protein in the environment. These overestimations could potentially have impacts on the risk

assessments for transgenic crops and insecticide formulations containing Cry proteins. Although validating ELISA results with bioassays is ideal, Western blotting and liquid chromatography – mass spectrometry also can be used to validate results generated by ELISAs.

### *Bioassays*

The best way to determine if biological activity of the Cry proteins remains is to perform bioassays on environmental samples with an insect species susceptible to the protein of interest. There are many different bioassay methods, depending on the sample matrix to be studied. Soil samples may be laid over the top of prepared insect diet or incorporated directly into the diet (115,123). Alternatively, Cry proteins may be extracted from soil samples and then the extract can be incorporated into the diet or overlaid on top of the diet (124). Plant tissue and detritus can be analyzed by direct feeding on the tissue, or by incorporating the tissue or detritus into the diet (37,125). Water samples may be analyzed by placing the insects directly into the water (92). After a pre-determined incubation period on the sample-infused diet, insect mortality and other parameters, such as insect weight or head capsule width, may be recorded to determine lethal and sub-lethal effects. Bioassays are only semi-quantitative; exact protein concentrations are impossible to define, but based on known  $LC_{50}$  and  $EC_{50}$  values (the concentration needed to cause mortality or a specific effect in 50% of the sample population, respectively), a general idea of the concentrations present can be determined. Other drawbacks that limit the usefulness of bioassays for biological validation are that they are time-consuming, expensive, and labor-intensive to set up and analyze, as well as to maintain insect colonies for further studies. Also, results may be skewed if an increase in

sample material (soil, plant tissue) in the diet is needed to increase protein concentrations to levels sufficient to cause negative effects. This increase in sample material may cause a decrease in the essential nutrients needed for insect survival, which could result in higher than anticipated mortality or effect levels.

The three studies discussed below give a sampling of how bioassays have been used for biological validation. Head *et al.* (126) analyzed soil samples for the presence of Cry1Ac from cotton with ELISAs and bioassays. Bioassays were performed by mixing soil with water to form a slurry, which was then mixed with an agar-based diet. After the diet solidified, one first-instar *Heliothis virescens* larva was introduced into each well. Larval survival and insect weights were determined after seven days. The bioassays supported the ELISA results that indicated no protein was present in any of the soil samples (126).

Shan *et al.* (115) analyzed soil samples for the presence of Cry1F from maize with ELISAs and bioassays. Rhizosphere soil samples were collected and diluted by a factor of 10 (weight:volume) with agar. This suspension was then laid over the top of previously prepared insect diet and one neonate *H. virescens* was placed in each well. Mortality and insect weights were recorded after six days. Cry1F was not detected in any of the rhizosphere soil samples by bioassays, which corroborated the ELISA results (115).

Zwahlen *et al.* (125) analyzed maize detritus collected from litter bags over a period of several months for the presence of Cry1Ab with ELISAs and bioassays. In the first year of the study, maize detritus was incorporated directly into the insect diet and fed to neonate *O. nubilalis*; mortality and insect weights were recorded after six days. In the

second year, maize detritus was mixed with extraction buffer and then added to the insect diet; mortality and insect weights were recorded after five days. In both years, larval mortality decreased over time. This finding supported the ELISA results, which showed that the Cry1Ab concentration in maize detritus also decreased over time (125).

### *Western blotting*

Western blotting is a technique used to identify specific proteins in a sample. First, gel electrophoresis is used to separate proteins and fragments in a sample by size. The proteins and fragments are transferred, or electroblotted, onto a nitrocellulose or polyvinylidene difluoride membrane. Antibodies specific to the protein of interest are incubated with the membrane and bind to the protein. The detecting antibody usually has an enzyme conjugated to it, similar to ELISA; in some cases, the enzyme is conjugated to a secondary antibody, which then binds to the detecting antibody. Finally, a substrate is added which allows for visualization of the protein bands on the membrane. Depending on the type of enzyme conjugated to the antibody and the type of substrate used, detection can be colorimetric, chemiluminescent, radioactive, or fluorescent (127). Unlike bioassays, western blotting is capable of detecting small quantities of protein in a sample. However, western blotting is still time-consuming, expensive, and not quantitative. These drawbacks are likely a few of the reasons western blotting is not used more often.

In addition to the studies described above, two other studies also have used Western blotting to validate their results. Gruber *et al.* (128) traced the fate of Cry1Ab protein in transgenic maize, through animal feed, and into liquid manure. Cry1Ab was detected by ELISA in transgenic maize and animal feed prepared from transgenic maize;

Western blotting confirmed the presence of fully intact protein. In the liquid manure samples, ELISAs showed a decrease in Cry1Ab concentration overtime, which was confirmed by Western blotting; however, after 24 weeks of storage, ELISAs still showed that Cry1Ab was present, while Western blotting detected only a 34 kDa fragment (128).

Paul *et al.* (129) analyzed gastrointestinal tract (GIT) samples from cows fed transgenic Cry1Ab maize or non-transgenic isoline maize. The ELISA results echoed the Einspanier *et al.* (121) and Lutz *et al.* (119) studies; Cry1Ab appeared to accumulate as it moved through the GIT. Western blotting determined that small quantities of fully intact Cry1Ab were present in all samples, but that the accumulation of protein observed in the ELISA results could be attributed to an increase in fragments approximately 17, 34, and 42 kDa in size, and not due to a increase in fully intact Cry1Ab (129).

#### *LC/MS*

Liquid chromatography paired with mass spectrometry (LC/MS) is another analytical method that may be used to detected Cry proteins in environmental samples. In this method, proteins are digested into peptides via proteolytic enzymes. These peptides are separated by high efficiency nanocolumn liquid chromatography, which feeds the peptides directly into the mass spectrometer. The peptides are ionized by the mass spectrometer and all intact peptide ions are measured. The instrument then selects peptide ions based on pre-determined criteria such as charge state or mass-to-charge ratio ( $m/z$ ) and subjects these selected peptides to collisionally induced dissociation (CID), which causes the peptide ions to fragment in a predictable manner. The CID fragmentation pattern can be used to determine the sequence of the peptide; this sequence can be compared to databases containing sequences of known proteins to identify the protein in

the sample and determine if it is fully intact or a fragment. This method allows for identification of a single protein or fragment in a complex mixture of proteins without the need for further purification (130).

Currently, the study of Cry proteins with LC/MS has been limited to identification of new toxins and investigations into the mode of action of Cry proteins. Yang *et al.* (131) utilized 2-dimensional liquid chromatography – tandem mass spectrometry to analyze protein samples from *Bacillus thuringiensis* strain 4.0718. The authors identified more than 1,000 unique proteins; eleven of these proteins were determined to be insecticidal Cry proteins. Bayyareddy *et al.* (132) used LC/MS to identify aminopeptidases and alkaline phosphatases, which are known receptors for Cry4Ba, in the detergent-resistant membranes (also known as lipid rafts) of *Aedes aegypti*. These detergent-resistant membranes had previously been suggested as potential entry points for bacterial pathogens and their toxins. No studies using LC/MS to analyze environmental samples or validate ELISA results were found in this current literature review. This is not surprising as the high initial set-up costs, maintenance costs, specialized training required to operate the instrument, and long sample analysis time (>90 minutes in both 131-132) make LC/MS a less favorable option for detection of Cry proteins and validating ELISA results in environmental samples.

## **Critical Review of Published Studies**

The final section of this article reviews several published studies and critiques their usage of ELISAs for detection of Cry proteins in the environment. This is not meant to be an exhaustive review of all published articles, but rather is intended to provide the reader with a general assessment of how the scientific community is utilizing ELISAs for



Cry protein detection and quantification and identify areas for improvement. Table 1 summarizes the reviewed articles.

### *Sensitivity*

The sensitivity of ELISAs is one of the areas of validation that is most commonly reported in research articles. The limit of detection (LOD) is most often reported, while the lower limit of quantitation (LLOQ) is reported less often. A few studies reported decision limits instead of LLOQ; these decision limits were determined according to governmental criteria (103,128,133). Additionally, a few studies reported only the LOD for the commercial ELISA kit used in the study, and did not report the LOD or LLOQ for the different matrices investigated (134-135). Of the studies that reported LOD values, the results confirm that ELISAs are a sensitive methods of detection; the LOD in these studies ranged from 0.01 ng/g (0.01 ppb) to 4 µg/L(4 ppb) in soil and 2.1 ng/L (2.1 ppt) to 6 µg/L (6 ppb) in water (106,136-139).

One-fourth of the studies reviewed failed to report any validation of sensitivity; thus, the sensitivity of the recovery methods and ELISA procedures used in these studies is unknown. In at least one study, the authors reported that no Cry1Ab protein was detected in soil that was in contact with decaying transgenic maize tissue (104). Since the authors did not report any sensitivity data, it is impossible to determine if, in fact, no protein was present, or if Cry1Ab protein was present in the soil, but the recovery methods and ELISA procedures used in the study were not sensitive enough to detect the protein.

### *Specificity*

The use of commercial ELISA kits targeted for detection of only one or two proteins has helped resolve some of the specificity issues that occur in environmental fate studies, especially when multiple Cry proteins may be present. However, cross-reactivity of the antibodies with components in the sample matrix or interference of the sample matrix, preventing antibodies from binding to Cry proteins, still needs to be addressed, regardless of whether or not a commercial kit is used. For example, Shan *et al.* (115) used a commercial ELISA kit, and observed slight matrix effects in samples of soil extracts. To mitigate the matrix effects, a 2x dilution was used for all samples.

Several studies analyzed blank soil or water samples for the presence of Cry proteins in parallel with their analysis on environmental samples. In nearly all of those control samples, no positive detections occurred; thus, cross-reactivity with sample matrix components was excluded. However, these blank samples do not account for the ability of matrix components to interfere with the quantitation of a protein. Testing for interference (as well as cross-reactivity) should be performed by mixing a 2x standard curve with the blank sample matrix (water, soil extract, etc.), resulting in a 1x standard curve in a 2x dilution of the matrix. Using a 1x standard curve prepared in assay buffer as a reference, the differences between the theoretical and observed values for the points in the standard curve prepared in the matrix can be calculated. Differences of greater than 20% may indicate interference or cross-reactivity, though this may vary (112). Another problem with running only blank matrix samples can be observed in Wang *et al.* (140). In that study, the authors detected protein in soil samples from plots planted with non-Bt rice. Since no further sensitivity validation steps were performed, it is difficult to

determine if the positive detection is the result of a basal level of protein in the soil, as the authors suggest, or if there was cross-reactivity to soil components (140).

As with sensitivity, one-fourth of the studies reviewed failed to report any validation of specificity. Therefore, the extent of the effect, if any, that cross-reactivity and interference of other proteins and matrix components may have on the results of these studies is difficult to ascertain.

### *Accuracy*

One-third of the studies reviewed failed to report any recovery or extraction efficiency values for accuracy. Thus, it becomes difficult to determine how efficient these extraction methods were at recovering Cry proteins from various environmental matrices. Further, of the studies that do include recovery values, many of the recovery values are significantly below the acceptable recovery range of 70-120%. Recovery values of 10-50% in soil are common, although higher recoveries are attainable (105,128,139,141). Soil type has a significant impact on the recovery values of Cry proteins observed in soil samples. Soils high in clay and silt content typically yield poor recovery of Cry proteins (10-50%) while soils high in sand content yield better recoveries (75-98%)(103,115,141). Recovery of Cry proteins in water also is highly variable, ranging from 23% to 78% (92,139). One study avoided this issue by analyzing the water directly; however, since no protein was detected in the water samples, extracting the samples (i.e., concentrating) would have been advisable to increase the sensitivity of the method (75).

Low recovery values are a known problem with extraction of Cry proteins from environmental matrices (78). One mechanism to manage this issue is to perform spike and recovery on multiple samples and determine the variation between the samples. A

coefficient of variation (%CV) of less than 20% provides support that a researcher is using an accurate extraction method, even though recovery of the protein may be less than 70% (112). Only one of the studies reviewed provided %CV values; Shan *et al.* (115) reported %CV values of 5.4-13.8% for soil recovery. An additional four studies reported recovery means and standard deviations (126,128,139,141). Thus, the %CV for these studies can be calculated by dividing the standard deviation by the mean, and multiplying by 100%. In these studies, the calculated %CV values range from 4.7 to 21.2% depending on the sample matrix. Three additional studies reported means, standard error, and sample size (101,138,142). Standard deviation in these studies can be calculated through the equation  $standard\ error = \frac{standard\ deviation}{\sqrt{sample\ size}}$ , or written another way,  $standard\ deviation = standard\ error \times \sqrt{sample\ size}$ . In these three studies, the calculated %CV values ranged from 6.1 to 35.1%. Eight of the reviewed studies provided the standard error or percentage recovery, but did not provide enough additional information (*i.e.*, sample size) to calculate %CV for the studies. As noted above, the remainder of the studies failed to even report percentage recovery.

### *Precision*

Precision is the area of validation reported the least in the literature reviewed here. Only six of twenty-six studies reported any information on validation of assay precision. Two studies used assay methods that previously had been validated for precision (137,139). Shan *et al.* (115) investigated precision of the assay across analysts and days (%CV = 9.4-14.6%), while Gruber *et al.* (128) validated the intra- and inter-assay precision (%CV = 5.9% and 14.6%, respectively). The final two studies reanalyzed plates that had a %CV less than 10% (75,101).

Three-fourths of the studies not reporting precision data did utilize commercial ELISA kits. Use of commercial kits is advantageous, as these kits undergo rigorous testing by the manufacturer during development to ensure uniformity with plates, between plates, across antibody lots, etc. Thus, some of the concerns regarding assay precision can be mitigated by the use of a commercial ELISA kit, while other areas still need to be validated (i.e., variation between analysts, etc.). Finally, five studies prepared ELISA plates in their laboratories using lab-generated or commercial sources of antibodies (92,103,125-126,133). All of these studies failed to report precision data. This is significant as the processes for preparing these plates in research labs is most likely not as refined as the processes used in commercial manufacturing facilities, which could result in inconsistencies across plates or even within a single plate. Additionally, there may be significant variability between lots of lab-generated antibodies. All of these factors can affect the variability of the assays, making comparisons between samples on separate plates difficult.

### *Biological Validation*

A vast majority of the studies failed to perform any form of biological validation. Thus, it is impossible to determine if the Cry proteins detected in environmental samples were fully intact and/or biologically active. A couple of papers recognized that the protein detected may not be fully intact and/or biologically active. Baumgarte and Tebbe (105) recognized that they could not “claim that the immunoreactive Cry1Ab protein detected in soils and plant residues was actually biologically active.” Nguyen and Jehle (143) also acknowledged that “it is not clear whether these ELISA detectable Cry1Ab residues still retain their bioactivity.” Gruber *et al.* (133) did not perform biological

validation, but protein was not detected in any of the soil samples, rendering biological validation unnecessary.

Seven papers performed bioassays with an insect species that was susceptible to the protein of interest in the study. In five of the studies, the bioassay results confirmed the ELISA results. In Zwahlen *et al.* (125) and Fejes *et al.* (92), the bioassay results showed decreasing mortality and sublethal effects as the protein levels, as determined by ELISA, also decreased. Bioassays performed by Head *et al.* (126) and Shan *et al.* (115) indicated that no Cry protein was present in any of the samples, which corresponded to the ELISA results. Wang *et al.* (142) performed bioassays on soil samples from the rhizosphere region of transgenic rice plants and observed no significant effects, confirming the ELISA results; however, the authors did not perform bioassays on samples taken from soil amended with transgenic rice tissue.

The remaining two studies produced the most interesting results. Bioassays performed by Marchetti *et al.* (144) suggested toxicity of the proteins decreased more rapidly than estimated by ELISA results. This is interesting because it indicates that the ELISAs may be detecting non-bioactive forms of the protein. Gruber *et al.* (128) biologically validated their results with Western blotting instead of bioassays. The results showed that Cry1Ab fragments of 17, 34, and 42 kilodaltons (kDa), as well as the full-size 65 kDa parent molecule, were detected in transgenic plant tissue and animal feed from transgenic plants. Only the 34 kDa fragment and the full-size protein were detected in liquid manure. The most interesting result, however, is that after 24 weeks of storage, the full-size protein had been degraded and only the 34 kDa fragment remained, yet Cry1Ab could still be detected in the slurry by ELISA at a concentration of

approximately 1 µg/g. This is interesting because, as with the Marchetti *et al.* (144) study, it indicates that the ELISA may be detecting degraded, non-bioactive forms of the protein.

## **Conclusions**

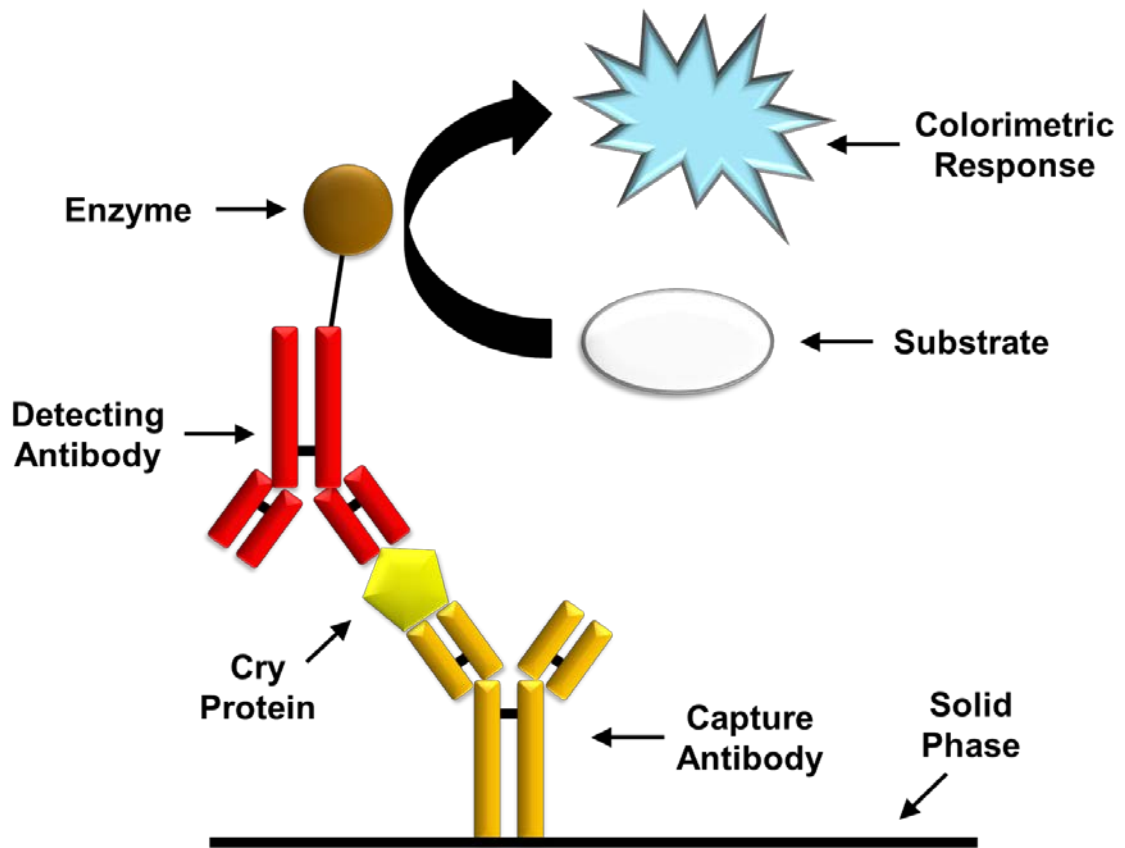
ELISAs have emerged as the predominant method for detecting Cry proteins in the environment. ELISA validation methods are necessary to ensure usable data are generated in environmental fate studies. These methods, and their associated acceptance criteria, have been validated through years of research, but they have yet to be widely adopted by environmental fate researchers. More than 90% of the studies reviewed failed to perform one or more of the five key areas of validation. Thus, substantial improvement in estimating the concentrations of Cry proteins in environmental samples can be made by simply increasing the practice of utilizing validation methods. Increasing the accuracy of environmental measurements will increase the usefulness of these data to regulators and will allow for a more efficient regulatory process.

## **Dissertation Organization**

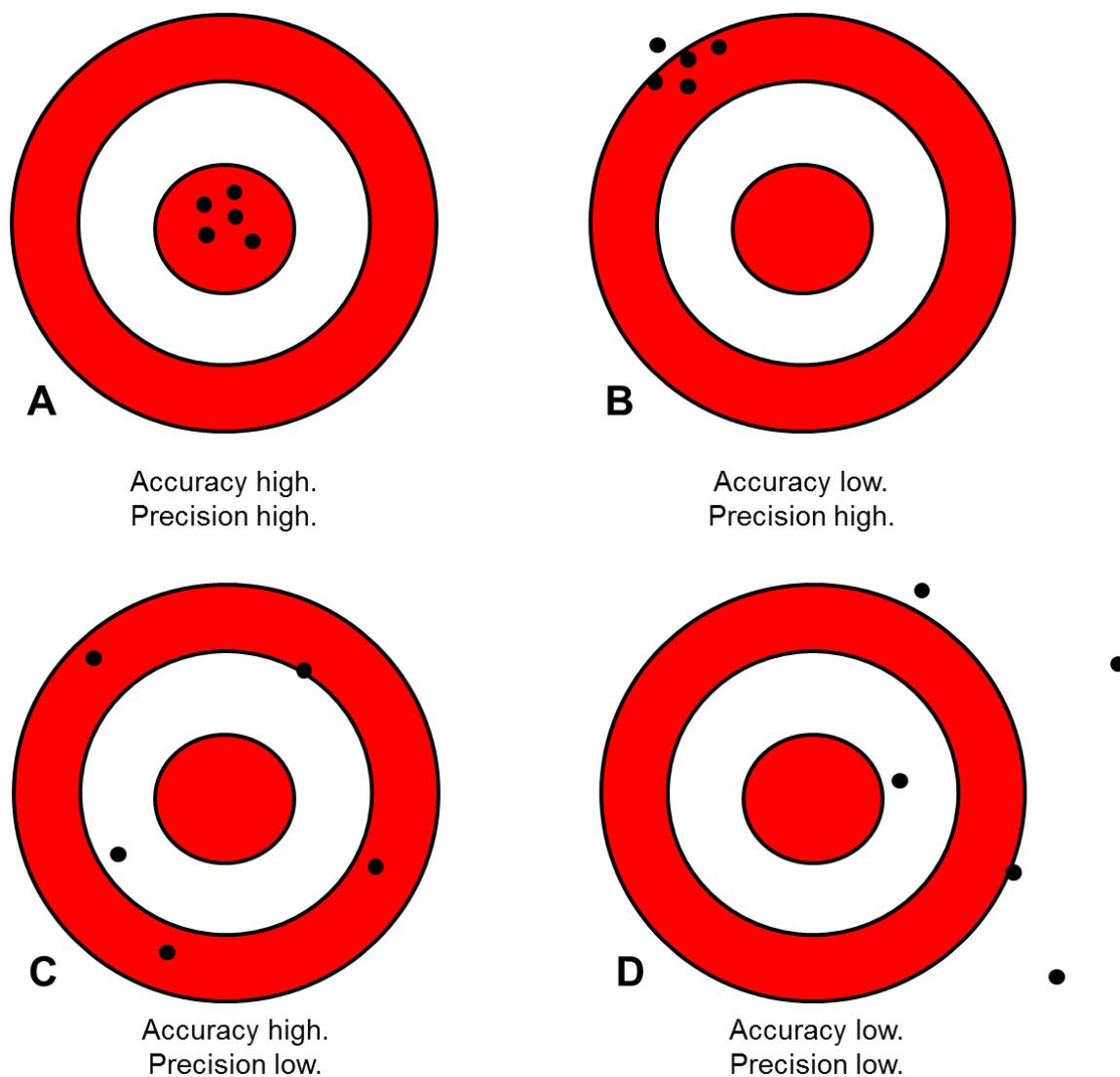
This dissertation is organized into four chapters to investigate the fate of Cry proteins in the environment. The first chapter provides background information on Cry protein history and usage, and detection of the proteins with ELISAs. It also provides a review of studies utilizing ELISAs for detection of Cry proteins in environmental samples and critiques their usage of validation methods. The second chapter describes enzyme and non-enzyme based model systems that were screened for their ability to generate fragments of Cry1Ab. The third chapter analyzes the fragments generated in the second chapter with ELISAs and bioassays to determine if the fragments are still detectable and/or if the fragments retain any biological activity. Finally, chapter 4 provides the overall conclusions that can be drawn from this research.



## Figures



**Figure 1.** Schematic of a typical direct sandwich ELISA.



**Figure 2.** Visual representation of accuracy and precision. The central ring, or bull's-eye, represents the true amount of target analyte in a sample and the individual dots represent sample replicates. **A.** All sample replicates are contained within the central circle; the results are both reproducible and accurate. **B.** Replicates are grouped tightly (reproducible), but provide an inaccurate result. **C.** Replicates are widely dispersed (not reproducible), but the average provides an accurate result. **D.** Replicates are neither accurate nor reproducible.

## Tables

**Table 1.** List of manufacturers of ELISA kits for detection of Cry proteins.

<b>Manufacturer</b>	<b>Target Protein(s)</b>
Agdia (Elkhart, IN, USA)	Cry 1Ab/Cry 1Ac Cry 1Ab/Cry 3Bb 1 Cry 1Ac/Cry 2Ab Cry 1F Cry 1F/Cry 34Ab 1 Cry 2A Cry 2A/Cry 3Bb 1 Cry 3A mCry 3A Cry 3Bb 1 Cry 34Ab 1
Creative Diagnostics (Shirley, NY, USA)	Cry 1Ab/Cry 1Ac Cry 1Ab/Cry 3Bb 1 Cry 1F Cry 1F/Cry 34Ab 1 Cry 2A Cry 3A Cry 3Bb 1 Cry 34Ab 1
Envirologix (Portland, ME, USA)	Cry 1Ab/Cry 1Ac Cry 1Ab/Cry 3Bb 1 Cry 1Ac/Cry 2Ab Cry 1C Cry 1F Cry 1F/Cry 34Ab 1 Cry 2A mCry 3A Cry 3Bb 1 Cry 34Ab 1 Cry 9C
Fitzgerald (Acton, MA, USA)	Cry 1Ab/Cry 1Ac
Romer Labs (Union, MO, USA)	Cry 1Ab Cry 1Ac Cry 1F

**Table 2.** Summary of the ELISA validation steps performed in studies on the environmental fate of Cry proteins.

Reference	Protein	Matrix	Sensitivity	Specificity	Accuracy	Precision	Biological Validation
Head <i>et al.</i> (123)	Cry1Ac	Soil	LOD = 3.68 ng/g; LLOQ not reported	Analyzed soils from non-Bt fields in parallel, no false positives, indicates cross-reactivity not significant; interference not reported	Mean recoveries ranged 24-39% depending on soil type; %CV = 21.2% <sup>1</sup>	Generated ELISA plates in-house; data on variation between or within plates or across days and plate lots not reported	Performed bioassays with a susceptible species; bioassays results indicated no protein was present in all samples, which confirmed the ELISA results
Hopkins and Gregorich (142)	Cry1Ab	Soil	LOD, LLOQ not reported	Cross-reactivity and interference due to matrix components not reported	No recovery methods or data reported efficiency of extraction procedure unknown	Used commercial ELISA kit; additional precision data not reported	Not performed; unknown if protein detected was fully intact and/or biologically active
Zwahlen <i>et al.</i> (122)	Cry1Ab	Maize detritus	LOD, LLOQ not reported	Analyzed maize detritus from non-Bt plants in parallel, no false positives, indicates cross-reactivity not significant; interference not reported	Extraction efficiency for maize detritus not reported	Generated ELISA plates in-house; data on variation between or within plates or across days and plate lots not reported	Performed bioassays with a susceptible species; bioassay results indicated decreasing mortality and sub-lethal effects over time, confirms ELISA results
Ahmad <i>et al.</i> (131)	Cry3Bb1	Soil	Kit LOD = 1 ng/mL; LOD, LLOQ for soil recovery method not reported	Analyzed soils from non-Bt fields in parallel, no false positives, indicates cross-reactivity not significant; interference not reported	Mean recoveries ranged 17-66% depending on spiking solution and soil type; standard error reported, but insufficient information to calculate %CV	Used commercial ELISA kit; additional precision data not reported	Not performed, unknown if protein detected was fully intact and/or biologically active

**Table 2.** (*continued*)

Reference	Protein	Matrix	Sensitivity	Specificity	Accuracy	Precision	Biological Validation
Baumgarte and Tebbe (102)	Cry1Ab	Soil, maize tissue	LOD = 0.19 ng/g soil after adjusting for recovery percentage; LLOQ not reported	Analyzed soils and maize tissue from non-Bt fields in parallel, no false positives, indicates cross-reactivity not significant; interference not reported	Recovery values ranged 34-40% in soil; standard error reported, but insufficient information to calculate %CV; extraction efficiency for maize tissue not reported	Used commercial ELISA kit; additional precision data not reported	Not performed; unknown if protein detected was fully intact and/or biologically active; authors recognized this data gap
Wang <i>et al.</i> (140)	Cry1Ab	Rice tissue, soil	LOD = 0.5 ng/g soil, not reported for rice tissue; LLOQ not reported	Analyzed non-Bt rice tissue in parallel, no false positives, indicates cross-reactivity not significant; cross-reactivity and interference in soil not reported	Mean recoveries ranged 46-82% depending on soil type; %CV ranged from 6.1-35.1% <sup>2</sup> depending on soil type; extraction efficiency for rice tissue not reported;	Used commercial ELISA kit; additional precision data not reported	Performed with susceptible species for rhizosphere soils, no significant effects observed, confirms ELISA results; not performed for soil amended with rice tissue, unknown if protein detected was fully intact and/or biologically active
Marchetti <i>et al.</i> (141)	Cry1Ab, Cry1Ac	Soil	LOD, LLOQ not reported	Cross-reactivity and interference due to matrix components not reported	Mean recoveries of 67% in clay loam soil, 82% in sandy soil; %CV not reported	Used commercial ELISA kit; additional precision data not reported	Performed with a susceptible species; bioassay results indicated that the decrease in toxicity occurred more rapidly than shown by ELISA results

**Tale 2. (continued)**

Reference	Protein	Matrix	Sensitivity	Specificity	Accuracy	Precision	Biological Validation
Nguyen and Jehle (139)	Cry1Ab	Maize tissue, detritus	LOD = 0.14 ng; LLOQ not reported	Analyzed non-transgenic, isoline maize in parallel, no false positives, indicates cross-reactivity not significant; interference not reported	Extraction efficiency from maize tissue not reported	Used commercial ELISA kit; additional precision data not reported	Not performed; unknown if protein detected was fully intact and/or biologically active, authors recognized this data gap
Gruber <i>et al.</i> (130)	Cry1Ab	Soil	LOD = 0.4 ng/g soil; Decision Limit (2 ng/g soil) and Detection Capacity (3.1 ng/g soil) determined according to European Commission Decision 2002/657/EC34	Analyzed soils from non-Bt fields in parallel, no false positives, indicates cross-reactivity not significant; interference not reported	Mean recoveries ranged 45-91% depending on soil type %CV not reported	Generated ELISA plates in-house; data on variation between or within plates or across days and plate lots not reported	Not performed, but no protein detected in any soil samples potentially renders bioassays unnecessary
Icoz and Stotzky (132)	Cry3Bb1	Maize tissue, root extracts, soil	Kit LOD = 1ng/mL; LOD, LLOQ for soil recovery method not reported	Cross-reactivity and interference due to matrix components not reported	Recovery from soil and root extracts and extraction efficiency from maize tissue not reported	Used commercial ELISA kit; additional precision data not reported	Not performed; unknown if protein detected was fully intact and/or biologically active
Prihoda and Coats (98)	Cry3Bb1	Soil, maize tissue	LOD = 0.7 ng/mL; LLOQ = 9 ng/g soil	Analyzed non-transgenic, isoline maize in parallel, no false positives, indicates cross-reactivity not significant; interference not reported	Mean recovery decreased from 41% (0 hr) to 15% (96 hr); %CV ranged from 6.1-35.1% <sup>2</sup> ; extraction efficiency from maize tissue not reported	Used commercial ELISA kit; reanalyzed plates with %CV >10%	Not performed; unknown if protein detected was fully intact and/or biologically active

**Table 2. (continued)**

Reference	Protein	Matrix	Sensitivity	Specificity	Accuracy	Precision	Biological Validation
Prihoda and Coats (72)	Cry3Bb1	Water, sediment, maize tissue	LOD, LLOQ not reported	Analyzed non-transgenic, isoline maize in parallel, no false positives, indicates cross-reactivity not significant; interference not reported	Water analyzed directly; recovery from sediment and extraction efficiency from maize tissue not reported	Used commercial ELISA kit; reanalyzed plates with %CV >10%	Not performed; unknown if protein detected was fully intact and/or biologically active
Schrader <i>et al.</i> (143)	Cry1Ab	Soil, maize detritus, earthworm tissue	LOD = 0.017 ng/g soil, 1 ng/g maize detritus, 0.58 ng/g earthworm tissue; LLOQ not reported	Cross-reactivity and interference not reported for any of the matrices investigated	Recovery from soil and earthworm tissue and extraction efficiency from maize detritus not reported	Used commercial ELISA kit; additional precision data not reported	Not performed; unknown if protein detected was fully intact and/or biologically active
Shan <i>et al.</i> (112)	Cry1F	Soil	LOD, LLOQ determined empirically for each soil type	Analyzed solutions of soil extracts for matrix effects; Used a 2x dilution to mitigate matrix effects	Mean recoveries ranged 66.9-90.5% depending on soil type; %CV ranged from 5.4-13.8%	Used commercial ELISA kit; tested method with 2 analysts across 2 days; %CV = 9.4-14.6%	Performed with a susceptible species; bioassays indicated no protein present, confirmed ELISA results
Daudu <i>et al.</i> (101)	Cry1Ab	Soil, maize tissue	LOD, LLOQ not reported	Cross-reactivity and interference due to matrix components not reported	Recovery from soil and extraction efficiency from maize tissue not reported	Used commercial ELISA kit; additional precision data not reported	Not performed; unknown if protein detected was fully intact and/or biologically active

**Table 2.** (*continued*)

Reference	Protein	Matrix	Sensitivity	Specificity	Accuracy	Precision	Biological Validation
Badea <i>et al.</i> (133)	Cry1Ab	Soil, maize tissue	LOD = 0.01 ng/g soil; LLOQ not reported	Analyzed soil free of Cry protein in parallel, no false positives, indicates cross-reactivity not significant; interference not reported	Mean recovery less than 40% in soil; %CV not reported; extraction efficiency from maize tissue not reported	Used commercial ELISA kit; additional precision data not reported	Not performed; unknown if protein detected was fully intact and/or biologically active
Miethling- Graff <i>et al.</i> (134)	Cry3Bb1	Soil, maize detritus	LOD = 0.01ng/g soil; LLOQ not reported	Analyzed soils from non-Bt plots in parallel, no false positives, indicates cross-reactivity not significant; interference not reported	Recovery from soil and extraction efficiency from maize detritus not reported	Used commercial ELISA kit that had been previously validated (Nguyen <i>et al.</i> 2008)	Not performed; unknown if protein detected was fully intact and/or biologically active
Tank <i>et al.</i> (103)	Cry1Ab	Maize detritus, water	LOD = 0.56 ng/mL (maize detritus) and 6 ng/L (water); LLOQ not reported	Used buffer blanks to account of buffer matrix effects; Did not account for matrix effects from maize detritus and water samples	Recovery from water and extraction efficiency from maize detritus not reported	Used commercial ELISA kit; additional precision data not reported	Not performed; unknown if protein detected was fully intact and/or biologically active
Gruber <i>et al.</i> (125)	Cry1Ab	Maize tissue, soil, animal feed, liquid manure	LOD = 0.4 ng/g; Decision Limit and Detection Capacity determined according to European Commission Decision 2002/657/EC34	No cross-reactivity or interference observed in any of the blank matrix samples analyzed	Mean recoveries ranged 68-98% depending on sample matrix; %CV ranged from 4.5-16.7% <sup>1</sup>	Intra-assay precision = 5.9% CV; Inter-assay precision = 14.6% CV	Western blotting used to determine protein fragmentation; ELISA results indicated protein in slurry at 24 weeks, but only fragments observed with Western blots



**Table 2. (continued)**

Reference	Protein	Matrix	Sensitivity	Specificity	Accuracy	Precision	Biological Validation
Helassa <i>et al.</i> (135)	Cry1Aa	Soil	LOD = 4 µg/L; LLOQ not reported	No interference due to soil matrix	Mean recoveries ranged 53-66% depending on soil type; %CV ranged from 9.0-29.4% <sup>2</sup> depending on soil.	Used commercial ELISA kit; additional precision data not reported	Not performed; unknown if protein detected was fully intact and/or biologically active
Shu <i>et al.</i> (144)	Cry1Ab	Earthworm tissue, soil	LOD, LLOQ not reported	Cross-reactivity and interference to soil and earthworm tissue not reported	Recovery from soil and earthworm tissue not reported	Used commercial ELISA kit; additional precision data not reported	Not performed; unknown if protein detected was fully intact and/or biologically active
Fejes <i>et al.</i> (89)	Cry4	Water	LOD = 2 ng/mL pure protein, 170-900 ng/mL in samples concentrated via lyophilization; LLOQ not reported	Matrix effects observed in undiluted Bti formulations used in standard curves; matrix effects of field-collected water used in study not reported	Mean recoveries 23-30%; insufficient information to calculate %CV	Used commercial antibodies, but prepared own microplates and antibody-enzyme conjugates; variability between plates, antibody-enzyme batches not reported	Performed with a susceptible species; bioassays indicated increased larval survival as concentration of protein decreased (concentration of protein determined by ELISAs)
Gruber <i>et al.</i> (100)	Cry1Ab	Soil	LOD = 0.4 ng/g soil; Decision Limit (2 ng/g soil) determined according to European Commission Decision 2002/657/EC34	Analyzed soils from non-Bt fields in parallel, no false positives, indicates cross-reactivity not significant; interference not reported	Mean recoveries ranged 49-89% depending on soil type; %CV not reported	Generated ELISA plates in-house; data on variation between or within plates or across days and plate lots not reported	Not performed; unknown if protein detected was fully intact and/or biologically active

**Table 2. (continued)**

Reference	Protein	Matrix	Sensitivity	Specificity	Accuracy	Precision	Biological Validation
Wang <i>et al.</i> (137)	Cry1Ab/1Ac fusion protein	Soil, root exudates, water	LOD 0.1ng/g (soil) and 0.005 ng/mL (water); LLOQ not reported	Analyzed water from non-Bt plots in parallel, no false positives, indicates cross-reactivity not significant; protein detected in soils from non-Bt plots	Mean recoveries ranged 46-82% depending on soil type; %CV not reported; recovery from root exudates not reported	Used commercial ELISA kit; additional precision data not reported	Not performed; unknown if protein detected was fully intact and/or biologically active
Whiting <i>et al.</i> (136)	Cry1Ab Cry3Bb1	Soil, sediment, water	LOD = 0.86 ng/g soil, 2.1 ng/L water LLOQ not reported	Previously validated (Mueting <i>et al.</i> 2014, Strain <i>et al.</i> 2014)	Mean recoveries of 77% in soil and sediment, 78% in water; %CV ranged from 9.1-17.9% <sup>1</sup>	Previously validated (Mueting <i>et al.</i> 2014, Strain <i>et al.</i> 2014)	Not performed; unknown if protein detected was fully intact and/or biologically active
Xue <i>et al.</i> (138)	Cry3Bb1	Soil, maize detritus	LOD, LLOQ not reported	Analyzed soils from non-Bt plots in parallel, no false positives, indicates cross-reactivity not significant; cross-reactivity and interference in maize detritus not reported	Mean recoveries ranged 10-96% depending on soil type; %CV ranged from 2.5-20.7% <sup>1</sup> ; extraction efficiency for maize detritus not reported	Used commercial ELISA kit; additional precision data not reported	Not performed; unknown if protein detected was fully intact and/or biologically active

<sup>1</sup>%CV calculated using mean and standard deviation provided by the authors.

<sup>2</sup>%CV calculated using mean, standard error, and sample size provided by the authors.

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## **CHAPTER 2. DEVELOPMENT OF A MODEL SYSTEM APPROACH FOR GENERATING FRAGMENTS OF THE CRY1AB PROTEIN**

*A paper to be submitted to the Environmental Toxicology and Chemistry*

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### **Abstract**

The use of transgenic crops expressing one or more Cry proteins for insect management has grown dramatically since their introduction nearly two decades ago. However, many questions surrounding the environmental fate of these proteins still persist. One area of particular interest is the possible detection of Cry protein fragments by the antibodies used in ELISA kits. A model system approach is used to generate environmentally relevant fragments. Eight different types of model systems were screened for their ability to generate fragments of the Cry1Ab protein; five of these model systems reliably generated Cry1Ab fragments. These fragments were analyzed in a subsequent study to determine if the fragments are still detectable by ELISA and if they retain any bioactivity.

### **Introduction**

Insecticidal crystal (Cry) proteins isolated from the bacterium *Bacillus thuringiensis* are widely used for insect pest management in agriculture. Since their introduction in 1996, transgenic maize expressing one or more of these Cry proteins now accounts for 81% of all maize planted in the United States [1]. Such widespread use has

led to questions regarding the environmental fate of these Cry proteins, including persistence, movement, and stability, in various environmental matrices.

Many researchers are addressing these questions by attempting to detect and quantify the amount of Cry proteins in environmental samples. A variety of detection methods are used to monitor for Cry proteins in environmental samples, but the most commonly used method is enzyme-linked immunosorbent assays (ELISA) [2]. Microtiter plate ELISAs are preferred to methods such as liquid chromatography/mass spectrometry (LC/MS), as the ELISA kits typically include all required materials; a large number of samples can be processed in a short amount of time (4-8 hr) with little specialized training, making them very cost-effective for researchers in academia and industry [2].

Several manufacturers produce ELISA kits for detection of Cry proteins, primarily for seeds and leaf tissue. Before these ELISA kits are used for detection of Cry proteins in environmental samples, the entire analytical method (including extraction protocol) must be validated. A quality validation study addresses the following: 1) sensitivity – defining the quantitative range and determining the upper and lower limit of quantitation; 2) specificity – determining that only the protein of interest is detected and there is no cross-reactivity or interference with related proteins or components in the matrix; 3) accuracy – ensuring the amount of protein detected in the ELISA is close to the actual amount in a sample; 4) precision – ensuring that the results are repeatable across days, analysts, and laboratories. These steps are well-researched and common in the literature [3-5].

Despite these steps, biological validation, i.e., ensuring the proteins detected are bioactive, is usually lacking. This is a crucial oversight, as a majority of the ELISA kits

utilize polyclonal antibodies. Polyclonal antibodies are produced by injecting the antigen of interest into a vertebrate host organism and later purifying the antibodies out of the serum. The process results in a heterogeneous mixture of antibodies that bind to multiple epitopes on a protein, increasing the chances of detection, and leading to very sensitive assays. However, use of polyclonal antibodies also increases the likelihood of a false positive result through the detection of a partially degraded protein. Although some antibody-binding sites on a partially degraded protein may be lost, other antibodies in the heterogeneous mixture may still be capable of binding to the protein fragment. This could result in an ELISA test indicating a positive detection in a sample where no fully intact protein exists. False positive results may lead to overestimations of the concentrations of these proteins in the environment, which potentially could impact the risk assessment for these proteins.

This phenomenon of Cry protein fragments producing false positive results is well known. Einspanier *et al.* [6] fed transgenic maize to cattle to trace select proteins through the bovine gastrointestinal tract (GIT) using a commercially available ELISA kit. They reported that Cry1Ab protein accumulated during the pass through the GIT; cross-reactivity with other proteins was not the cause, as the effect was not observed in cattle fed isoline, non-transgenic maize [6]. In a subsequent study, the authors hypothesized that the protein was fragmented, yet still immunoactive, leading to its detection [7]. This hypothesis was tested by feeding transgenic maize containing Cry1Ab to cattle, collecting samples from the GIT at slaughter, and analyzing them with ELISAs and Western blotting. The ELISA results indicated that the Cry1Ab concentration increased during passage through the GIT. However, the Western blotting results showed that no Cry1Ab



was present in any samples; only bands of approximately 34 and 17 kDa were observed, whereas the source Cry1Ab was 60 kDa, calling into question the ELISA results [7]. Therefore, it is crucial to understand how fragmented proteins affect ELISAs and to determine if any bioactivity is retained by these fragments.

In order to determine if Cry protein fragments are still detectable by ELISAs, a reliable and reproducible method of generating Cry protein fragments must first be developed. The objective of this study is to use a model system approach to degrade Cry1Ab protein into environmentally relevant fragments. A model system approach is useful in that it allows researchers to control all parameters in a system; one to a few parameters can then be altered to determine the effects changes would have on the system as a whole. In this study, use of a model system approach allows Cry1Ab to be degraded in a controlled manner and produce environmentally relevant fragments. Five enzyme-based model systems using the enzymes trypsin, chymotrypsin, pepsin, proteinase K, and a simulated gut fluid are evaluated. Non-enzyme based model systems, including sunlight/UV photodegradation, an acidic buffer only, and long-term degradation in buffer were also tested for their ability to generate fragments of Cry1Ab. Gel electrophoresis with Coomassie or silver staining was used to confirm fragmentation of the protein. Bioassays were performed on model system components to ensure that these components would not have a significant effect on insect survival or weight gain.

## **Methods and Materials**

### *Gel Electrophoresis*

Polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE) was used to screen model systems for production of Cry1Ab fragments. Gels were

prepared fresh daily according to a previously described procedure [8]. Recipes in Table 1 were modified from Rosenberg [8] to fit a Bio-Rad Mini-Protean II gel apparatus (gel size 83 mm x 73 mm x 1 mm). Samples were prepared for electrophoresis by adding an appropriate amount of 4X sample buffer to obtain a 1X sample buffer concentration. Sample buffer was prepared by combining 4 g sucrose, 0.8 g sodium dodecyl sulfate (SDS), 2.5 mL 1M Tris-HCl (pH 6.8) and 250  $\mu$ L 0.1% (w/v) bromophenol blue and diluting to 10 mL with nanopure water. Samples were heated to 100°C for 3-4 min, chilled in ice for 10 min and centrifuged at 12,000 x g for 5 min. Running buffer (3.03 g Tris (hydroxymethyl)aminomethane (THAM), 14.42 g glycine, 1 g SDS, diluted to 1 L with nanopure water) was added to the upper and lower chambers of the apparatus. A 20- $\mu$ L sample was added to each well. Each gel also contained 5  $\mu$ L of PageRuler™ Unstained Broad Range Protein Ladder (Fisher Scientific) with molecular bands ranging from 5 kilodaltons (kDa) to 250 kDa. Gels were run at a constant 180 volts until the dye front was within 1 centimeter of the bottom of the gel (approximate run times were 1 hr). Gels were then removed from the glass plates and stained with either Coomassie Brilliant Blue or silver stain. Gels were digitized using a Lexmark X83 All-in-One printer/copier/scanner and Lexmark Viewing Booth software (Lexmark International Inc., Lexington, KY). Images of the gels were edited and annotated using Adobe Photoshop CS5 (version 12.1 x64) (Adobe Systems Inc., San Jose, CA).

#### *Coomassie Staining*

The Coomassie staining procedure from Rosenberg [8] was used. Briefly, Coomassie stain was prepared by dissolving 1 g Coomassie Brilliant Blue R-250 (Fisher Scientific) in 100 mL acetic acid and 400 mL methanol and diluting to 1 L with nanopure

water. Destain solution was prepared by diluting 100 mL acetic acid and 400 mL methanol to 1 L with nanopure water. Gels were stained in Coomassie stain for 30 min and destained with destain solution for 2-3 hr or overnight with the destain solution being changed 1-2 times. All staining steps took place on an orbital shaker (ELMI Ltd., Riga, Latvia) at 100 rpm.

### *Silver Staining*

The silver staining procedure from Rosenberg [8] also was used. Briefly, after gel electrophoresis, gels were stored in gel fix solution (50% (v/v) methanol, 12% (v/v) acetic acid, 38% (v/v) water) overnight. Gels were washed three times with 50% (v/v) ethanol for 20 min each. Gels were then submerged in sodium thiosulfate (0.2 g/L sodium thiosulfate) for exactly 1 min and then rinsed with nanopure water three times for 20 sec each. Gels were submerged in silver nitrate solution (2 g/L silver nitrate, 0.75 mL/L 37% (v/v) formaldehyde stock solution) for 20 min and rinsed with nanopure water 2 times for 20 sec each. Bands were visualized by submerging gels in developing solution (60 g/L sodium carbonate, 0.5 mL/L 37% (v/v) formaldehyde stock solution, 4 mg/L sodium thiosulfate) for up to 10 min. The developing process was terminated by washing gels with nanopure water twice for 2 min each. Finally, gels were submerged in gel fix solution for 10 min and then in 50% (v/v) methanol for 20 min. All steps were performed at room temperature on an orbital shaker.

### *Bioassays*

Insect diet was prepared by mixing one part Stonefly *Heliothis* diet (Ward's Science, Rochester, New York) with three parts liquid, containing treatment or control solution. The diet was thoroughly mixed, and 0.3 mL of diet was placed into each well.

There were 16 wells per replicate and 3 replicates per treatment (N=48). One European corn borer (ECB) neonate, *Ostrinia nubilalis*, was placed in each well and incubated at 27°C ( $\pm$  2°C) and 50-65% humidity. European corn borers were provided by the Corn Insects and Crop Genetics Research Unit of the USDA–ARS, Ames, IA. After seven days, larvae were assessed for survival and weight gain. Statistical significance ( $p < 0.05$ ) was determined using an analysis of variance in SAS 9.3 software (SAS Institute, Cary, NC).

#### *Range-Finding Bioassays*

Range-finding bioassays were used to determine the LC<sub>90</sub> and LC<sub>95</sub> values specific to the protein and insect colony used in the study. The protein used in this study was trypsin-activated Cry1Ab protein (salt-free, 96% pure) dissolved in N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) buffer and was purchased from Case Western Reserve University (Cleveland, OH). The concentration of the stock solution was 900 µg/mL.

Two preliminary range-finding bioassays were performed to determine an appropriate range for the bioassays. The first preliminary bioassay used eight concentrations with a range of 1-500 ng/g diet, and the second preliminary assay narrowed the range to 5-125 ng/g diet. A water control and buffer control that consisted of equal parts 100 mM potassium phosphate, pH 7.6 (enzyme solution buffer) and 50 mM CAPS, pH 10.5 (protein solution buffer) were included in each bioassay. From the two preliminary bioassays, the concentrations of Cry1Ab used in the range-finding experiment were set at 15, 30, 45, 60, 75, 90, 105, and 120 ng/g diet. Five range-finding

bioassays were started on five different days. A probit analysis in SAS 9.3 was used to analyze the data and determine the LC<sub>90</sub> and LC<sub>95</sub> values.

### *Buffer Determination*

A variety of buffers were screened with bioassays to determine if any of the buffer components affected insect survival or weight gain. Table 2 lists buffer composition and enzyme compatibility. A total of 7 different buffers were tested using the previously described bioassay procedure.

### *Trypsin*

Trypsin was the first enzyme tested for the ability to degrade Cry protein. Trypsin is a serine protease found in the gastrointestinal tract of vertebrate and invertebrate animals. Vertebrates, such as cattle fed on transgenic maize, and invertebrates, such as detritivores feeding on maize detritus, may excrete Cry protein fragments into soil and water where they may be detected by ELISAs. Additionally, trypsin also acts as a surrogate for other serine proteases, which are ubiquitous in eukaryotes and prokaryotes. All enzymes were tested using an enzyme:toxin (mol:mol) ratio [9]. In order to terminate the model system at a specific time point, an appropriate enzyme inhibitor that has no significant effects on insect survival and weight gain is needed. Trypsin from bovine pancreas (cat. no. T9201), trypsin inhibitor from *Glycine max* (cat. no. 93620), and N $\alpha$ -benzoyl-L-arginine-p-nitroanilide hydrochloride (BAPA)(cat. no. B3133) were purchased from Sigma-Aldrich (St. Louis, MO) and were used in an activity assay to determine the amount of inhibitor needed to completely inhibit the activity of trypsin. The activity assay used was modified from Smith *et al.* [10]. Four types of sample tubes were prepared as described in Table 3: reagent blank, standard, sample blank, and sample. The substrate

solution was prepared by dissolving 10 mg BAPA into 50 mL of potassium phosphate buffer ( $\text{KH}_2\text{PO}_4$ ) that was heated to 37°C. The concentration of trypsin used in the model system and activity assay was a 5:1 mol:mol ratio trypsin:toxin dissolved in  $\text{KH}_2\text{PO}_4$  buffer. A wide range of inhibitor concentrations (1-1000  $\mu\text{g/mL}$ ) dissolved in  $\text{KH}_2\text{PO}_4$  buffer was used to determine a narrow range (50-250  $\mu\text{g/mL}$ ) for use in the activity assay. Absorbance at 410 nm was recorded for each sample and sample blank, and corrected absorbance was determined by subtracting the appropriate blank from the corresponding sample. Then, change in absorbance was calculated by subtracting each of the corrected sample absorbance values from the corrected standard absorbance. Finally, percentage inhibition was calculated by dividing the change in absorbance from each sample by the corrected standard absorbance and multiplying by 100%.

To ensure neither the trypsin nor the inhibitor have any detrimental effects on insect survival and growth, bioassays were performed with a water control, a buffer control, a 5:1 trypsin solution, a 200- $\mu\text{g/mL}$  inhibitor solution, and the trypsin and inhibitor combined according to the previously described bioassay procedure.

The trypsin model system consisted of three samples: Cry1Ab control, trypsin and inhibitor control, and a treatment (Table 4). At termination, 150  $\mu\text{L}$   $\text{KH}_2\text{PO}_4$  (Cry1Ab control) or 150  $\mu\text{L}$  200  $\mu\text{g/mL}$  trypsin inhibitor in  $\text{KH}_2\text{PO}_4$  buffer (trypsin and inhibitor control, treatment) was added to each vial. Four time points were used, 10 min, 1 hr, 8 hr, 24 hr, and each was performed in duplicate for a total of six vials per time point. All vials were held at 37°C for the duration of the experiment. An additional two Cry1Ab control vials held at room temperature also were added for each time point, to determine if the elevated temperature produced any degradation of the protein. After termination, all

samples were flash frozen with liquid nitrogen and stored at -80°C until analysis with gel electrophoresis.

### *Chymotrypsin*

Alpha-chymotrypsin from bovine pancreas (cat. no. C4129) trypsin-chymotrypsin inhibitor from soybean (cat. no. T9777), and N-glutaryl-L-phenylalanine-p-nitroanilide (GPANA) (cat. no. G2505) were purchased from Sigma-Aldrich. Like trypsin, chymotrypsin is a serine protease that can act as a surrogate for other serine proteases. Chymotrypsin also is found in the gastrointestinal tract of vertebrate and invertebrate organisms. An activity assay modified from the previously described trypsin assay was performed to determine the amount of inhibitor needed to completely inactivate chymotrypsin. Four types of sample tubes were prepared as described in Table 5. Substrate solution was prepared by dissolving 27 mg GPANA into 2 mL methanol while gently heating and then diluting to 70 mL final volume with 68 mL  $\text{KH}_2\text{PO}_4$ . Two concentrations of chymotrypsin were used: a 50:1 and 5:1 mol:mol ratio by dissolving chymotrypsin into  $\text{KH}_2\text{PO}_4$  buffer. Two concentrations were chosen to compensate for lowered chymotrypsin activity compared to trypsin activity ( $\geq 40$  units/mg and  $\geq 7,500$  units/mg, respectively). Inhibitor concentrations of 500-1500  $\mu\text{g/mL}$  and 50-250  $\mu\text{g/mL}$  were used for the 50:1 and 5:1 ratios respectively. Absorbance at 410 nm was recorded for each sample and sample blank, and corrected absorbance was determined by subtracting the appropriate blank from the corresponding sample. Then, change in absorbance was calculated by subtracting each of the corrected sample absorbance from the corrected standard absorbance. Finally, percentage inhibition was calculated by

dividing the change in absorbance from each sample by the corrected standard absorbance and multiplying by 100%.

To ensure neither the chymotrypsin nor the trypsin-chymotrypsin inhibitor have any detrimental effects on insect survival and growth, bioassays were performed with a water control, a buffer control, a 50:1 chymotrypsin solution, a 1300- $\mu\text{g/mL}$  inhibitor solution, a 5:1 chymotrypsin solution, a 200- $\mu\text{g/mL}$  inhibitor solution, and the two combinations of chymotrypsin and inhibitor according to the previously described bioassay procedure.

The chymotrypsin model system consisted of a Cry1Ab control, and a chymotrypsin control and a treatment for each of two concentrations of chymotrypsin (50:1 and 5:1 solutions)(Table 6). No inhibitor was utilized in the chymotrypsin controls or treatments because the presence of the inhibitor would make identification of fragments difficult (see discussion). Instead, inhibition of the samples was achieved by flash freezing the samples in liquid nitrogen. Three time points were used, 1, 8, and 24 hr. At termination, 150  $\mu\text{L}$  nanopure water was added to each vial. All vials were heated to 37°C for the duration of the experiment. After termination, all samples were flash frozen with liquid nitrogen and stored at -80°C until analysis with gel electrophoresis.

### *Pepsin*

Pepsin (cat. no. P6887) was purchased from Sigma-Aldrich. Pepsin is a proteolytic enzyme utilized in many digestibility studies and also serves as a surrogate for other aspartate proteases found in the environment. The substrate used in the activity assay, succinyl-albumin, was synthesized according to Furihata *et al.* [11]. An activity assay modified from Strugala *et al.* [12] was performed to determine the amount of



inhibitor needed to completely inactivate pepsin. Four types of sample tubes were prepared as described in Table 7. A 10 mg/mL-substrate solution was prepared by dissolving succinyl-albumin in 0.01M HCl. Pepsin was prepared by dissolving 1.86 mg into 10 mL  $\text{KH}_2\text{PO}_4$  (pH 2.2) (186  $\mu\text{g/mL}$ ). A 10% (w/v) sodium bicarbonate solution was used to terminate the reaction, and a 10 mM 2,4,6,-trinitrobenzenesulfonic acid (TNBS) solution was used for color development. Initially, five concentrations of sodium hydroxide were used: 0.05, 0.125, 0.25, 0.5, and 1 M NaOH. Complete inhibition was observed for all concentrations, so a second assay was performed using lower concentrations, 2.5, 5, 12.5, 25, and 50 mM NaOH. Absorbance at 490 nm was recorded for each sample and sample blank, and corrected absorbance was determined by subtracting the appropriate blank from the corresponding sample. Then, change in absorbance was calculated by subtracting each of the corrected sample absorbance values from the corrected standard absorbance value. Finally, percentage inhibition was calculated by dividing the change in absorbance from each sample by the corrected standard absorbance and multiplying by 100%.

To ensure neither the pepsin nor the inhibitor have any detrimental effects on insect survival and growth, bioassays were performed with a water control, a buffer control ( $\text{KH}_2\text{PO}_4$ , pH 2.2), a 5:1 mol:mol pepsin:toxin solution, a 60-mM NaOH inhibitor solution, and the pepsin and inhibitor combined according to the previously described bioassay procedure.

The pepsin model system consisted of four buffer controls, a pepsin and inhibitor control, and a treatment (Table 8). Two concentrations of pepsin were prepared, a 10:1 and a 5:1 mol:mol ratio pepsin:toxin, by dissolving the pepsin in 0.1 M  $\text{KH}_2\text{PO}_4$ , pH 1.3.

A more acidic pH than used in the activity assay was necessary to compensate for the basic pH of the CAPS buffer in which the Cry1Ab protein was dissolved. The final pH of the solutions after mixing was 1.64.

In some preliminary experiments (not shown), Cry1Ab was degraded in the controls. To determine if this degradation was the result of the acidic buffer or the elevated temperature, four buffer controls were utilized. One control contained 75  $\mu$ L 50 mM CAPS, resulting in a basic pH (10.5). The other control consisted of 75  $\mu$ L Cry1Ab solution and 75  $\mu$ L  $\text{KH}_2\text{PO}_4$  (pH 1.3), resulting in an acidic pH of 1.64, which mimicked the treatments. One of each type of control was held at room temperature, while another of each was held at 40°C, for a total of four buffer controls. All controls were terminated with 150  $\mu$ L nanopure water; the subsequent increase in pH resulted in termination of the reaction. Samples were held at 40°C for 1 or 4 hr. There were two additional time points for the 10:1 concentration, 10 min and 30 min. All buffer controls were performed singularly, while the pepsin and inhibitor controls and the treatments were performed in duplicate. At termination, 150  $\mu$ L of water or NaOH were added (Table 8). After termination, all samples were flash frozen with liquid nitrogen and stored at -80°C until analysis with gel electrophoresis.

Based on the results, a second model system was performed with pepsin. This model system consisted of a basic and acidic buffer control (previously described), three pepsin and inhibitor controls (10:1, 5:1, and 1:1 mol:mol pepsin:toxin ratios) and three treatments (10:1, 5:1, and 1:1 mol:mol pepsin:toxin ratios). All samples were held at 40°C for 30 min or 1 hr. Only 1 replicate was performed for each sample. Following gel electrophoresis, gels were stained with silver stain instead of Coomassie stain. Silver

stain was used as it is more sensitive than a Coomassie stain, and it may allow for visualization of fragments that are missed by the Coomassie stain.

#### *Acidic buffer*

Due to the high degree of fragmentation observed in the acidic buffer controls, an acidic buffer model system was investigated for its ability to degrade Cry1Ab. Although the preliminary results suggested that this model system was useful in generating numerous fragments of various sizes, these fragments are probably less environmentally relevant than fragments generated by other model systems because the acidic conditions under which the protein fragmented are not widely found in agricultural settings. To ensure that the acidic buffer would not have any detrimental effects on insect survival and growth, bioassays were performed with a water control and an acidic buffer ( $\text{KH}_2\text{PO}_4$ , pH 1.3) treatment according to the previously described bioassay procedure.

The first acidic buffer model system consisted of four samples and three time points; as this was a range-finding study to determine optimal incubation times, no replication was performed. One sample contained 75  $\mu\text{L}$  Cry1Ab solution and 75  $\mu\text{L}$  50 mM CAPS, resulting in a basic pH (10.5). The other sample consisted of 75  $\mu\text{L}$  Cry1Ab solution and 75  $\mu\text{L}$   $\text{KH}_2\text{PO}_4$  (pH 1.3), resulting in an acidic pH of 1.64. One of each type of sample was held at room temperature, while another of each was held at 40°C, for a total of four samples per time point. All controls were terminated with 150  $\mu\text{L}$  nanopure water at 24, 36, or 48 hr. After termination, all samples were flash frozen with liquid nitrogen and stored at -80°C until analysis with gel electrophoresis.

A second acidic buffer model system was evaluated, extending the incubation time to 4-7 days. The basic pH samples were dropped from the study, leaving only acidic

samples at room temperature and 40°C, with the latter being performed in duplicate. Samples were terminated with 150 µL nanopure water at 4, 5, 6, and 7, days. After termination, all samples were flash frozen with liquid nitrogen and stored at -80°C until analysis with gel electrophoresis.

### *Proteinase K*

Proteinase K (cat. no. P6556) was purchased from Sigma-Aldrich. Proteinase K is the main proteolytic enzyme found in *Engyodontium album* (formerly *Tritirachium album*), a fungus that has been found in the cysts of soybean plants and found to be associated with reeds in wetland areas [13-15]. The buffer used for proteinase K was a 20 mM Tris-(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, pH 7.5 [16]. No activity assay was performed for proteinase K. Inhibition of the samples was achieved by flash freezing the samples in liquid nitrogen. To ensure neither the buffer nor the proteinase K enzyme have any detrimental effects on insect survival and growth, bioassays were performed with a water control, a buffer control, and a 1:1 mol:mol ratio proteinase K:toxin solution in Tris-HCl buffer according to the previously described bioassay procedure.

The proteinase K model system consisted of three samples: Cry1Ab control, proteinase K control, and a treatment (Table 9). Two time points were used, 1 hr and 24 hr, and the proteinase K and treatment samples were performed in duplicate. A second model system was set up using shorter time points and less proteinase K. For the second model system, the same three sample types were used (Table 9). Only one Cry1Ab control and one proteinase K control (containing a 1:1 ratio of proteinase K) were used for each time point, while two concentrations of proteinase K (a 1:1 ratio and a 0.5:1

ratio) were used in duplicate in the treatments. Three time points were used in the experiment: 15 minutes, 30 minutes, and 1 hr. All vials were heated to 37°C for the duration of the experiment. At termination for both model systems, 150 µL nanopure water was added to each vial. After termination, all samples were flash frozen with liquid nitrogen and stored at -80°C until analysis with gel electrophoresis.

#### *UV/Sunlight*

An ultraviolet (UV) model system was set up to investigate if exposure to shortwave UV rays would degrade Cry1Ab protein. Two control samples were prepared by combining 75 µL Cry1Ab solution, 75 µL CAPS buffer, and 150 µL nanopure water into two wells of a 96-well plate. This plate was placed on a lab bench and remained uncovered. Four treatment samples were prepared using the same solutions as the controls; however, these samples were combined in a UV transparent plate (Corning Inc., Corning, NY) and placed in a UV light box (Ultra-Violet Products Inc., San Gabriel, CA). This plate was exposed to shortwave UV rays (254nm) and all other sources of light were blocked out. Both plates remained at room temperature for the duration of the investigation. Nanopure water was added as needed in response to evaporation. One control and two treatment samples were collected at 12 hr and at 24 hr. Samples were flash frozen in liquid nitrogen and stored at -80°C until analysis with gel electrophoresis.

A second model system was set up to use natural light to attempt to degrade Cry1Ab. Three control samples and three treatment samples were prepared by combining 75 µL Cry1Ab solution and 225 µL CAPS buffer. The control samples were placed in separate wells in a non-UV transparent 96-well plate. The treatment samples were placed in a UV transparent 96-well plate. All wells were sealed with plastic wrap

and super glue to prevent evaporation. The control plate was wrapped in aluminum foil to completely block out any sunlight and both plates were laid out in direct sunlight on the roof of the National Laboratory for Agriculture and the Environment building in an area free from shadows. The experiment was started at 8:03 am (CDT) on July 28, 2014, and one control and one treatment sample were collected at 1, 4, and 8 hr by piercing the plastic wrap and pipetting out the solution. Solutions were transferred to 1.5-mL vials, flash frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until analysis with gel electrophoresis. The temperature was  $17.2^{\circ}\text{C}$  at the start of the experiment and was  $22.8^{\circ}\text{C}$  at the end with a high of  $23.2^{\circ}\text{C}$ . At the start of the experiment, there was no cloud cover, but conditions became mostly cloudy by hour 3 and remained that way for the duration of the experiment

([http://www.wunderground.com/history/airport/KAMW/2014/7/28/DailyHistory.html?req\\_city=Ames&req\\_state=IA&req\\_statename=Iowa](http://www.wunderground.com/history/airport/KAMW/2014/7/28/DailyHistory.html?req_city=Ames&req_state=IA&req_statename=Iowa)).

A third model system was set up to use an artificial UV light source. This artificial light was generated by using a Rayonet® photoreactor (Southern New England Ultraviolet Company, Branford, CT). This photoreactor uses eight lamps to produce a high intensity light source. Two different model systems were screened; one using lamps producing light at 365 nm ( $\pm 50$  nm) and one using lamps producing light at 254 nm. For both model systems, 1 mL of Cry1Ab solution was placed in a Pyrex test tube (for use with the 365 nm lamps) or a quartz cuvette (for use with the 254 nm lamps). The sample containers were then placed separately into the machine on a rotating carousel. A 75  $\mu\text{L}$  aliquot was removed and placed in separate 1.5 mL vials at each of the following time points: 5, 10, 15, and 30 minutes, and 1, 2, and 4 hr. After collection, 225  $\mu\text{L}$  of nanopure

water was added to each vial. A 50- $\mu$ L aliquot was prepared from each sample for gel electrophoresis, and all samples were flash frozen in liquid nitrogen and stored at -80°C until analysis by gel electrophoresis and silver staining.

#### *Simulated Gut Fluid*

A simulated earthworm gut fluid model system was adapted from Ma *et al.* [17]. Two concentrations of enzymes were used: a 5:1 ratio (containing 5 mol amylase and 5 mol cellulase to 1 mol Cry1Ab) and a 1:1 ratio (1 mol amylase and 1 mol cellulase to 1 mol Cry1Ab). There were two time points, 1 hr and 24 hr, and 4 vials per time point (Table 10). All vials were kept at 40°C for the duration of the study. At termination, 150  $\mu$ L nanopure water was added to each vial, and the samples were flash frozen in liquid nitrogen and stored at -80°C until analysis with gel electrophoresis.

#### *Long-term degradation in buffer*

A model system was set up to explore degradation of Cry1Ab protein over extended periods of time. Unlike previous model systems, this system was performed in triplicate. Each sample in the model system contained 100  $\mu$ L Cry1Ab solution and 100  $\mu$ L 50 mM CAPS. Four time points were used in the study and there were three replicates per time point: 4, 8, 12, and 16 weeks. All samples were stored in the dark at room temperature. At termination, 200  $\mu$ L of nanopure water was added to each vial and a 50  $\mu$ L aliquot was removed and placed in a separate vial for gel electrophoresis. All vials were flash frozen with liquid nitrogen and stored at -80°C until analysis by gel electrophoresis and silver staining.

## Results

### *Range-Finding Bioassays*

The results from the two preliminary bioassays were used to narrow the scope of concentrations for the range-finding bioassays (data not shown). The results of the range-finding bioassays are in Figure 1. The original experiment was performed with three replicates on different days. The second replicate had significantly lowered survival in the controls (survival <80% for both the water and buffer control), so a fourth replicate was performed. This replicate also had significantly lowered survival in the controls (survival <35% for both controls), and thus a fifth replicate was performed. Therefore, the LC<sub>90</sub> and LC<sub>95</sub> values were calculated using only the first, third, and fifth replicates. The LC<sub>90</sub> value was determined to be 82.4 ng protein per g insect diet, and the LC<sub>95</sub> value was determined to be 103.7 ng protein per g insect diet. A concentration of 94.5 ng/g was chosen for use in the model system degradations.

### *Buffer Determination*

Only one of the seven buffers, buffer 3, had no significant effects on insect survival or average weight gain (Figure 2). Of the remaining buffers, buffer 2 caused 100% mortality of the ECB larvae, and four buffers significantly decreased average weight gain, while one significantly increased average weight gain. Buffer 3 was compatible with all three of the enzymes to be tested initially, and thus it was used in the following trypsin, chymotrypsin, and pepsin studies.

### *Trypsin*

A preliminary activity assay using a wide range of inhibitor concentrations (1-1000 µg/mL) was used to establish a narrower range of concentrations for the activity



assay (data not shown). The narrow range used in the activity assay was 50-250  $\mu\text{g/mL}$ . Percentage inhibition was plotted, linear regression was performed, and the resulting equation was utilized to determine that a 147- $\mu\text{g/mL}$  concentration of trypsin inhibitor was needed for complete inactivation of trypsin (Figure 3). For ease of solution preparation and to provide a margin for error, a 200- $\mu\text{g/mL}$  inhibitor solution was used for all trypsin model systems. Bioassay results suggest that there were no significant differences in insect survival due to the presence of trypsin or the trypsin inhibitor (Figure 4). The presence of trypsin significantly decreased weight gain in larvae; however, this effect was not present in the trypsin-and-inhibitor treatment, indicating that the trypsin inhibitor from *Glycine max* used in the trypsin model system inhibited the activity of trypsin and prevented detrimental effects from occurring. Therefore, an assumption was made that any significant effects observed in bioassays with trypsin-degraded Cry1Ab protein was not the result of the presence of trypsin or the trypsin inhibitor.

No significant degradation of Cry1Ab was observed at any of the time points (Figure 5). Some minor degree of degradation was observed at 8 and 24 hr (Figure 5D) and in some of the controls, but this is more likely the result of freeze/thaw cycles or naturally occurring degradation over time than presence of trypsin. There was no difference between the room temperature Cry1Ab controls and the Cry1Ab controls held at 37°C.

#### *Chymotrypsin*

Percentage inhibition was plotted separately for the 50:1 and 5:1 activity assays, linear regression was performed, and the resulting equations were utilized to determine

that 1210- $\mu\text{g/mL}$  and 145- $\mu\text{g/mL}$  concentrations of trypsin-chymotrypsin inhibitor, respectively, were needed for complete inactivation of chymotrypsin (Figure 6). For ease of solution preparation and to provide a margin for error, 1300- $\mu\text{g/mL}$  and 200- $\mu\text{g/mL}$  inhibitor solutions were used for 50:1 and 5:1 chymotrypsin model systems, respectively. Bioassay results indicated that 50:1 ratio and its corresponding inhibitor concentration did not significantly affect insect survival, but it did have significant effects on average weight gain of the larvae (Figure 7A). The 5:1 ratio and its corresponding inhibitor concentration did not significantly affect insect survival (Figure 7B). While the 5:1 chymotrypsin and 200- $\mu\text{g/mL}$  inhibitor samples individually did not affect average weight gain, when combined, the average weight of the larvae after 7 days was significantly increased. Due to the presence of multiple bands in the controls on the trypsin gels, a test gel was run with samples of only 5:1 chymotrypsin, only 200- $\mu\text{g/mL}$  inhibitor, and the chymotrypsin and inhibitor together. The presence of numerous bands in all three wells made determination of Cry1Ab fragment bands from chymotrypsin and inhibitor bands difficult (Figure 8).

The chymotrypsin model system was performed without an inhibitor, based on the success of the proteinase K model system, which did not utilize an inhibitor. A similar degradation pattern was observed for both amounts of chymotrypsin used at all three time points: decreased amounts of parent protein were present and two distinct fragment bands were observed (Figure 9). The amount of parent protein appeared to decrease between 1 hr and 24 hr. The fragment bands also appeared to decrease over time, and no additional fragment bands were observed. The chymotrypsin bands also began to disappear at 24 hr,

suggesting that it also is being degraded and that longer incubation times would likely be of little use.

### *Pepsin*

For the first pepsin activity assay (NaOH concentrations 0.05-1 M), 100% inhibition was observed for all concentrations (data not shown). The activity assay was then repeated using NaOH concentrations of 2.5-50 mM. Percentage inhibition was plotted, linear regression was performed, and the resulting equations were utilized to determine that an inhibitor concentration of 49.6 mM NaOH was needed for complete inactivation of chymotrypsin (Figure 10). To provide a margin for error, a 60-mM NaOH solution was used for termination of all pepsin model system incubations.

Bioassay results indicate that the 5:1 pepsin solution and the 60-mM NaOH inhibitor have no effect on insect survival either individually, or when combined (Figure 11). Average weight of the insects in the buffer control and pepsin only treatments was significantly lower than the water control, inhibitor only, and pepsin-and-inhibitor combined treatments. This is most likely due to the acidic pH values in the diet. Average weight of the insects in the inhibitor only and pepsin-and-inhibitor combined treatments was significantly lower than the water control, but significantly higher than the buffer only and pepsin only treatments. The acidity of the inhibitor only and the pepsin and inhibitor combined treatments, while more basic than the buffer control and pepsin only treatments, were still acidic enough to have negative effects on insect growth.

A 10:1 pepsin concentration completely degraded all the Cry1Ab at 30 min, 1 hr, and 4 hr (Figure 12B-D). At 10 min, there was significant Cry1Ab degradation, with a little fully intact protein remaining (Figure 12A). A 5:1 concentration of pepsin also

degraded Cry1Ab substantially, leaving only a small amount of parent compound (Figure 13). None of the time points and pepsin concentration combinations produced observable Cry1Ab fragments. However, repeatable degradation was observed in the buffer controls with an acidic pH. The degree of degradation observed appears to be correlated with incubation time; longer time points (Figure 12C-D) exhibited more degradation than shorter time points (Figure 12A-B). A second model system was performed using one additional, lower concentration (1:1) and a silver staining technique. As with the previous model system, Cry1Ab was substantially degraded at all concentrations of pepsin and small amounts of parent compound remained (Figure 14). While the silver staining allowed visualization of more bands on the gel, no fragments were observed that could not be accounted for by the controls. The acidic buffer controls again showed increasing fragmentation of Cry1Ab with increasing incubation time.

#### *Acidic buffer*

Bioassay results suggest that the acidic buffer has no significant detrimental effects on insect survival or average weight gain (Figure 15). This is in contrast to the result observed in the pepsin preliminary bioassay where the buffer control (i.e., same acidic buffer used in this bioassay) significantly decreased average weight gain. A possible explanation for this difference is that there is inherent variability in any biological system, and increasing the number of insects would resolve the issue.

The first attempt at using only an acidic buffer to degrade Cry1Ab produced limited fragmentation (Figure 16). Almost no degradation was observed in any of the room temperature samples. Small amounts of degradation were observed in the 40°C samples, with the acidic buffer samples exhibiting more degradation than the basic

samples. However, a substantial amount of parent Cry1Ab still remained. The model system was repeated and extended to four-seven days. These extended time points exhibited significant degradation of the protein with nearly all the parent protein completely degraded after seven days (Figure 17). Although there was substantial degradation at all four time points, the number of different fragments and the quantity of each fragment were low. The most prominent band for all samples is a band near 5 kDa.

#### *Proteinase K*

Neither the Tris-HCl buffer nor the proteinase K enzyme had any significant effects on insect survival or weight gain (Figure 18). The first proteinase K model system screened two time points to determine if any degradation of Cry1Ab occurred. Complete degradation of the Cry1Ab protein was observed at both one and 24 hr, yielding only a single band at around 5 kDa (Figure 19). A second model system was performed using shorter time points and less proteinase K in an attempt to capture more or larger fragments. However, nearly complete degradation of Cry1Ab was again observed (Figure 20). This occurred even at the shortest time point and lowest proteinase K concentration.

#### *UV/Sunlight*

No significant degradation of the Cry1Ab protein occurred at either 12 hr or 24 hr (Figure 21) in the short-wave UV experiment. There were no differences between the 12-hr and 24-hr time points and the controls, suggesting that short wave UV rays alone are not enough to substantially degrade the protein. For the sunlight experiment, there was not a significant difference between the dark controls and the sunlight treatments at any time period (Figure 22). Further, there appears to be similar amounts of degradation at the 1-hr and 8-hr time points. In the photoreactor study, no significant degradation was

observed at any of the time points in the 365 nm treatment (Figure 23A). Under harsher conditions with the 254 nm lamps, some degradation was observed at the 5, 10, and 15-minute time points (Figure 23B). However, another phenomenon was also observed: at all seven time points, a band larger than 100 kDa was observed. By 30 minutes, all of the fully intact protein had dissipated, replaced by substantial quantities of a considerably larger aggregate, and small quantities of a small fragment or fragments approximately 5 kDa or smaller in size.

#### *Simulated Gut Fluid*

There was no significant degradation of Cry1Ab at either time point (Figure 24). Additionally, there was no difference in degradation between the two enzyme concentrations. Further, the enzymes themselves appeared to be mostly degraded after 24 hr. This earthworm-based system only included enzymes for degrading lipids and starches, so it is not surprising that the Cry1Ab protein was still intact after incubation.

#### *Long-term degradation*

Degradation of the Cry1Ab protein occurred at all time points, and there appears to be an increase in quantities of the fragments over time (Figure 25). Substantial amounts of the parent protein still remain, even at 16 weeks. There is one anomaly; replicate 3 of the 12-week samples has undergone more degradation than the other two replicates, resulting in additional fragments smaller than 15 kDa (Figure 25B). Since a similar pattern was not observed in any other samples, this degradation is most likely the result of contamination.

## Discussion

Lethal concentration values generated in bioassays can vary among insect populations, the type of bioassay (*i.e.*, overlay vs. incorporation), and, in the case of Cry toxin, the supplier of the toxin [18]. Therefore, it was important to determine the concentration of the Cry1Ab protein used in the study that would cause 90-95% mortality in the population of ECB to be used in the study. Normally, there is not much value in using LC<sub>90</sub> or LC<sub>95</sub> values due to the increase in variability away from the center of the curve (*i.e.*, the LC<sub>50</sub> value). However, in order to detect possible decreases in mortality as the Cry1Ab protein is degraded in this experiment, it is important to cause mortality in a large majority of the insects without using excessive amounts of Cry1Ab. Using the LC<sub>50</sub> value allows 50% of the insects in the experiment to survive, rendering them useless for the analysis. Likewise, using more protein than is needed could result in decreases in toxicity due to protein degradation being masked. By using a final concentration of 94.5 ng of protein per g insect diet for the bioassays, which falls between the LC<sub>90</sub> and LC<sub>95</sub>, the fully intact protein still causes significant mortality, while degraded proteins show decreasing mortality if they are no longer bioactive.

Trypsin did not significantly degrade the Cry1Ab protein (Figure 5). Trypsin is one of the proteolytic enzymes present in an insect midgut that is responsible for cleaving the Cry1Ab protoxin to produce the active form. Since the protein utilized in this study was already activated, it could be expected that further degradation would not be observed. This result is supported by Diaz-Mendoza *et al.* [9]. In that paper, the authors subjected a Cry1Ab protoxin to degradation by three purified trypsins. In all three treatments, only a 69 kDa fragment was produced, even after 24 hr. As a result of these

findings, it was determined that a trypsin-based model system would not be useful in generating Cry1Ab fragments.

Pepsin and its inhibitor had no effect on insect survival, but did have some effects on weight gain. While it would be ideal for components of a potential model system to have no effect on survival and weight gain, it was decided to move forward with the pepsin model system to see if detectable fragments could be generated. Ultimately, it did not matter that weight gain of the insects was affected, as pepsin significantly degraded Cry1Ab, but yielded no fragments that were detectable by gel electrophoresis. This effect was consistently observed despite numerous attempts to alter pepsin concentration and reduce incubation time (Figure 14). A change in staining technique from a Coomassie stain to a more sensitive silver stain produced additional bands in the controls, but did not generate any Cry1Ab fragments that were detectable by gel electrophoresis.

The current hypothesis is that pepsin (which cleaves between hydrophobic amino acids, such as tyrosine and phenylalanine) rapidly degrades Cry1Ab into amino acids or very small fragments that are not detectable by gel electrophoresis. This is most apparent at shorter time points (Figure 12A, 14A) where fully intact Cry1Ab protein remains, but no intermediate fragments are observed. As a result of these findings, it was determined that a pepsin-based model system would not be useful in generating Cry1Ab fragments. However, there were varying degrees of Cry1Ab degradation observed in the acidic buffer controls, suggesting that a model system based on an acidic buffer alone may be suitable for fragmenting Cry1Ab. An acidic buffer model system was tested over a range of incubation times to determine if prolonged exposure to an acidic environment would result in Cry1Ab degradation. Longer time points (4-7 days) were the best intervals at



generating fragments of Cry1Ab. The acidic buffer model system produced the highest number of different fragments, (4-8 bands on the gel [Figure 17]). Some parent Cry1Ab still remained after 4-7 days. This parent protein should still be detectable by ELISAs and have bioactivity; however, a decrease in absorbance (in the ELISAs) and bioactivity should be observed if the fragments are not detectable by ELISA. Further work is needed to determine the number of fragments present (particularly in the band near 5 kDa). This will be performed in a subsequent study (Chapter 3), which also will determine if the fragments are detectable by ELISAs and if any bioactivity is retained by the fragments.

An activity assay was performed with proteinase K; however, the background absorbance of the inhibitor, proteinase K enzyme, and substrate utilized were too high to generate any significant results (data not shown, see Appendix A). As the samples were flash frozen with liquid nitrogen immediately after termination, the hypothesis was that an inhibitor would not be needed. Without the use of the inhibitor, all concentrations of proteinase K yielded nearly complete degradation of Cry1Ab at all tested time points. Changing the incubation time or amount of proteinase K did not affect degradation, as the product in all of the samples was the same: a single band in the 5 kDa range. It is possible that the visualized band is not a single 5 kDa fragment, but rather a combination of multiple fragments that are 5 kDa or smaller. The limitations of the gel mixture used in the current study made it impossible to resolve the fragments in more detail; a 15% gel is ideal for separating and resolving proteins between 10-60 kDa [8]. As the goal of this study was to identify model systems that were capable of degrading Cry1Ab into fragments, no further work was performed with the fragments in the study. Separating and determining the number of fragments present will occur in a subsequent study

(Chapter 3), where the fragments generated also will be subjected to bioassays and ELISAs to determine if any of the fragments are still detectable and/or if any bioactivity is retained.

Initially, the chymotrypsin model system appeared not to be viable due to the presence of numerous bands from both chymotrypsin and the inhibitor. However, after the success of the proteinase K model system, in which no inhibitor was used, the chymotrypsin model system was revisited. Both levels of chymotrypsin significantly degraded Cry1Ab, producing two fragments that were visualized by gel electrophoresis (Figure 9). Longer time points produced decreasing amounts of parent Cry1Ab; however, no additional fragments were formed, and fragment and chymotrypsin abundance also decreased, suggesting further breakdown of both. There was no difference in degradation between the 50:1 and 5:1 mol:mol ratios of chymotrypsin:toxin. This is advantageous as it is best to avoid using the 50:1 ratio if possible due to the significant growth effects that were observed on insects fed diet containing that concentration of chymotrypsin (Figure 7A). Further work to determine if the fragments are detectable by ELISAs and if they retain any bioactivity was performed in a subsequent study (Chapter 3).

Degradation of proteins (including Cry proteins) subjected to UV light is known to occur, with cysteine, phenylalanine, tryptophan, and tyrosine being the primary sites of photodegradation [19-20]; however, no significant degradation was observed in this UV model system (Figure 21). This is not surprising as only a single UV wavelength was used and it was from a weak light source. In an attempt to generate environmentally relevant fragments, a model system was designed to use natural sunlight. While some degradation was observed in the samples, there was no difference between the controls

and treatments (Figure 22). A possible explanation for this is that the plastic wrap is not UV transparent and thus, it blocks UV rays from entering into the wells and degrading the protein. There was also no difference in degradation between the 1-hr and 8-hr time points. It is possible that most of the degradation observed occurred in the first hr of the experiment when direct sunlight was present. Further degradation may have been inhibited by cloud cover. This, however, is unlikely as UV rays can still penetrate cloud cover, and only heavy cloud cover can significantly reduce the amount of UV radiation reaching the earth's surface [21].

A final attempt to use UV light to degrade Cry1Ab utilized a photoreactor and two different wavelengths of light, 365 nm and 254 nm. The initial focus was on the 365 nm wavelength because it produces light within the natural solar spectrum and provides the most relevant results [22]. No degradation was observed at this wavelength (Figure 23A). This was unexpected as there are numerous reports of formulations of Bt insecticide being inactivated under natural sunlight [23-24]. The most likely explanation for this is that this study used pure Cry1Ab protein. Pure protein does not degrade readily in sunlight because the highest absorption band of pure protein is in the 280-285 nm range; the solar spectrum decreases to zero intensity in the 300-305 nm range [22]. Cry proteins in Bt insecticide formulations degrade readily in sunlight because the formulations often contain cofactors, prosthetic groups, or other adjuvants that can act as chromophores to capture photons and transfer energy to the Cry protein, resulting in its degradation [19, 22].

Since no degradation was observed at 365 nm, a harsher UV treatment, 254 nm, was used. Although this wavelength does not naturally occur, it may provide clues about

how Cry1Ab degrades under natural sunlight. Degradation of Cry1Ab occurs rapidly at 254 nm, with fragments visible after only 5 minutes (Figure 23B). However, exposure to UV light at 254 nm also appears to have other unintended consequences. At all time points, a majority of the protein was converted into a molecule greater than 100 kDa in size, and by 30 minutes nearly all of the protein was in this form. The most likely explanation is that the UV light caused the fully intact protein and/or protein fragments to become cross-linked to each other, forming dimers and other types of aggregate molecules; such protein-protein crosslinking is known to occur at 254 nm [25]. It is unknown if the presence of these dimers/polymers will lead to a potential increase or decrease in toxicity or if the aggregates are even detectable by ELISA. Despite the presence of cross-linked protein, a UV photodegradation model system is capable of generating fragments of Cry1Ab protein. Further research in a subsequent study (Chapter 3) at shorter time points will determine if the fragments and/or dimers are still detectable by ELISA and if they retain any bioactivity.

Simulated gastric systems were investigated for their potential to degrade Cry1Ab. However, most gastric systems use pepsin as the prominent enzyme, which has already been explored separately in this paper [26-28]. One model system identified as not using pepsin was simulated earthworm gut model system described in Ma *et al.* [17]. In Ma *et al.* [17], the authors developed three simulated earthworm gut formulations: an enzyme formulation (amylase and cellulase), a microbe formulation (cultured from the gut of *Eisenia andrei*) and an enzyme and microbe combination. They tested the ability of these three formulations to affect the bioaccessibility of arsenic, copper, and zinc in digested field soils. Their results showed that the enzyme formulation and the enzyme

and microbe combination produced the best results with little to no difference between the two. For the sake of simplicity, only the enzyme formulation was chosen to be used in this study. No degradation of Cry1Ab was observed in this model system (Figure 24). Additionally, the enzymes themselves were almost completely degraded after 24 hr. This is not unexpected as the digestions in Ma *et al.* [17] only lasted 3.5 hr, which is the approximate residence time of the gut contents of *E. andrei*. Due to a lack of promising results and the rapid degradation of the enzymes used in this model system, no further work was performed with this simulated gastric system.

Long-term degradation of Cry1Ab protein in CAPS buffer yielded low to moderate amounts of degradation, with substantial amounts of parent protein remaining, even after 16 weeks (Figure 25). This is in contrast to other studies where Cry1Ab has been reported to have half-lives of a few days [29-31]. However, these studies were performed in biologically active soil and water samples where microbes and other organisms are available to assist in the degradation of the protein. Although the model system components were not sterilized at the start of the experiment, there was no visual evidence that such organisms were present in the course of the study (i.e. the solution remained clear, and no algal or microbial films were observed on the surface of the solutions or the glassware). The 16-week samples were determined to exhibit substantial degradation. Further work to determine if the fragments are detectable by ELISAs and if they retain any bioactivity will be performed in a subsequent study (Chapter 3).

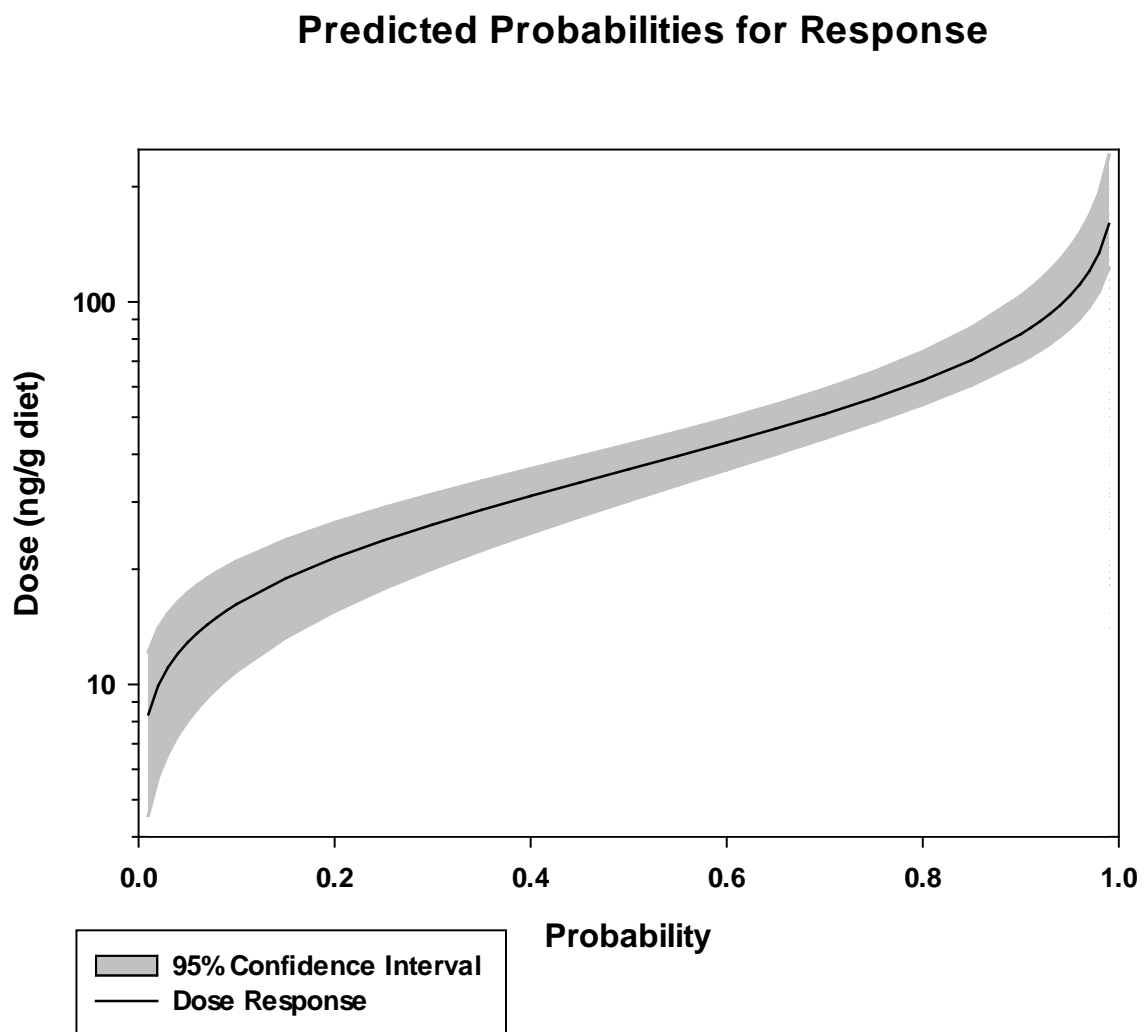
## **Conclusions**

Multiple approaches were utilized in attempts to degrade Cry1Ab into environmentally relevant fragments. Five model systems were identified that could

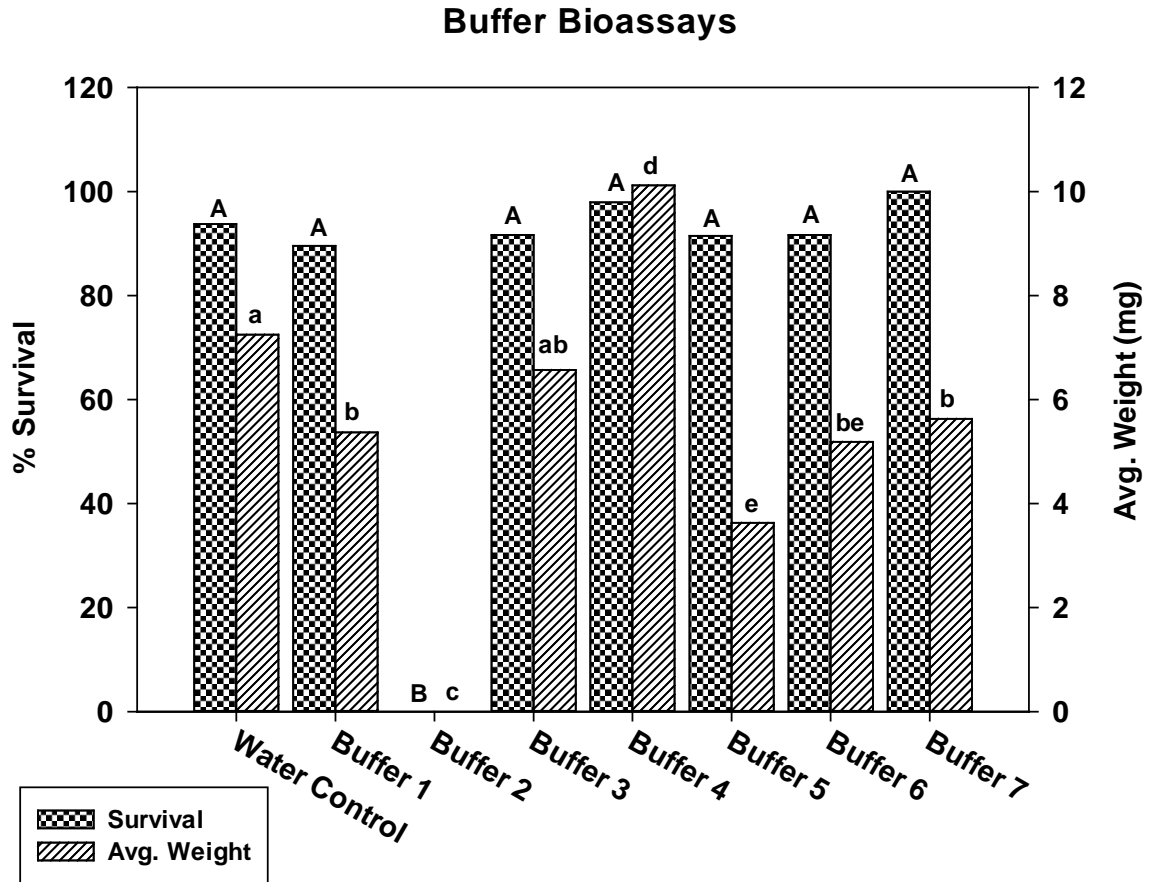
reliably generate Cry1Ab fragments. These five systems, chymotrypsin model system, proteinase K model system, acidic buffer model system, photodegradation model system, and long-term degradation model system are used in further studies (Chapter 3) to determine if fragmented proteins generated in environmental matrices are still detectable by ELISAs and if they retain any bioactivity.

## **Acknowledgements**

We thank Chad Boeckman and Kris Sturtz with DuPont Pioneer for providing training with the bioassay procedure and Keith Bidne with the U.S. Department of Agriculture – Agricultural Research Service Corn Insects and Crop Genetics Research Unit for kindly providing the European corn borer larvae used in the study. We thank William Jenks for access to the Rayonet photoreactor for the UV photodegradation studies. Mention of a proprietary product does not constitute an endorsement or a recommendation by Iowa State University or USDA for its use.

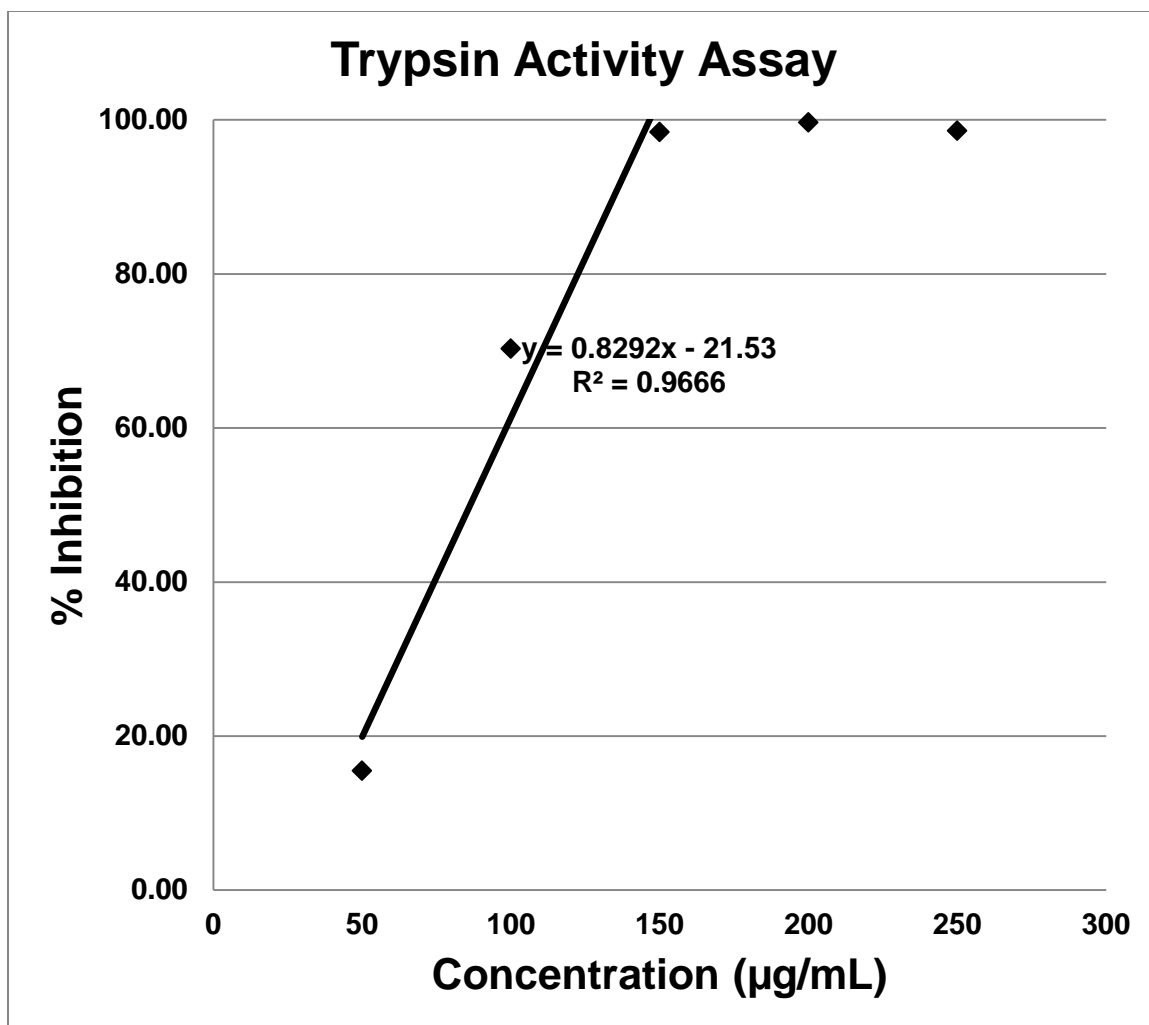
**Figures**

**Figure 1.** Range-finding bioassays. Due to the lowered control survival in replicates 2 and 4, only replicates 1, 3, and 5 were used to determine the  $LC_{90}$  and  $LC_{95}$  values of 82.4 ng/g and 103.7 ng/g, respectively.

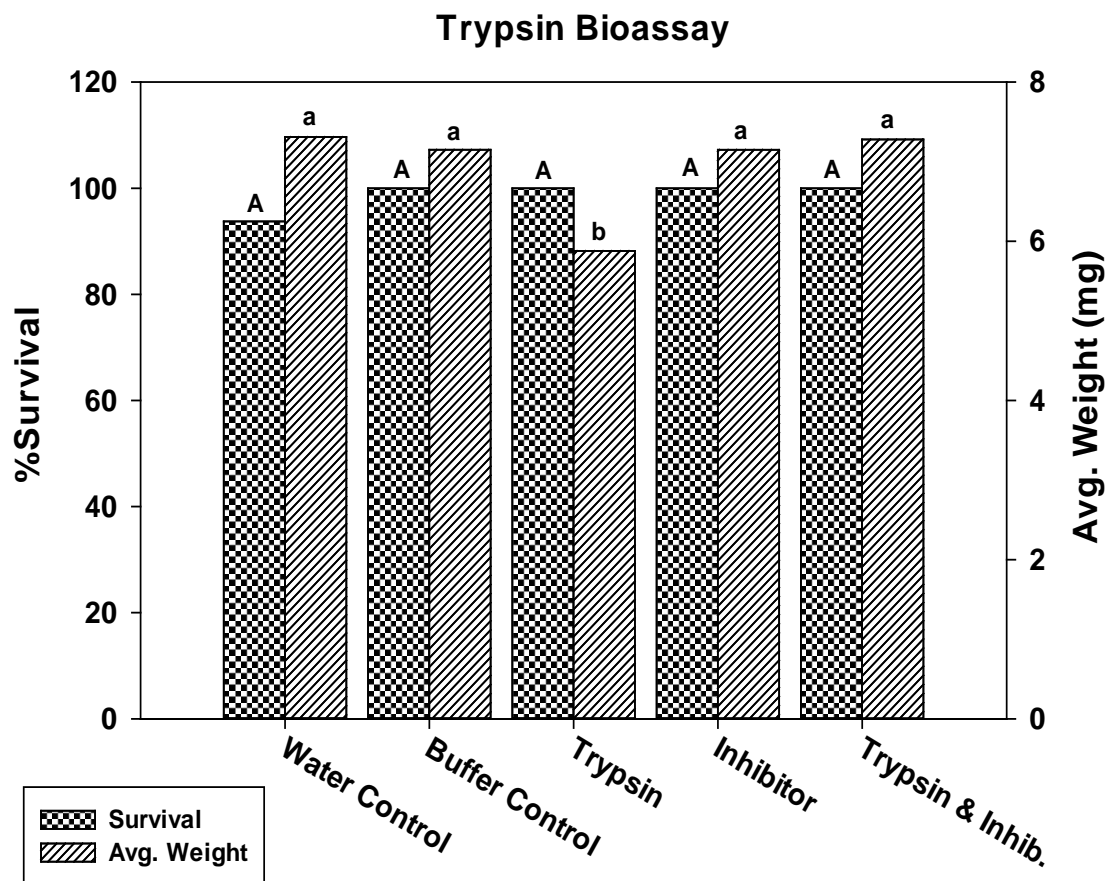


**Figure 2.** Buffer determination bioassays. Uppercase letters indicate significance within survival across buffers ( $p = 0.05$ ). Lowercase letters indicate significance within average weight across buffers ( $p = 0.05$ ). Buffer 1: 10 mM phosphate, pH 7.0-8.0. Buffer 2: 1 mM Tris-HCl, 1 M urea, 0.01% (w/v) SDS, pH 8.5. Buffer 3: 0.1 M potassium phosphate, pH 7.6. Buffer 4: 98 mM sodium bicarbonate, 2 mM sodium carbonate, pH 8.0. Buffer 5 0.1 M Tris-HCl, 10 mM calcium chloride, pH 7.8. Buffer 6: 50 mM HCl. Buffer 7: 50 mM KCl, 30 mM HCl, pH 1.5.

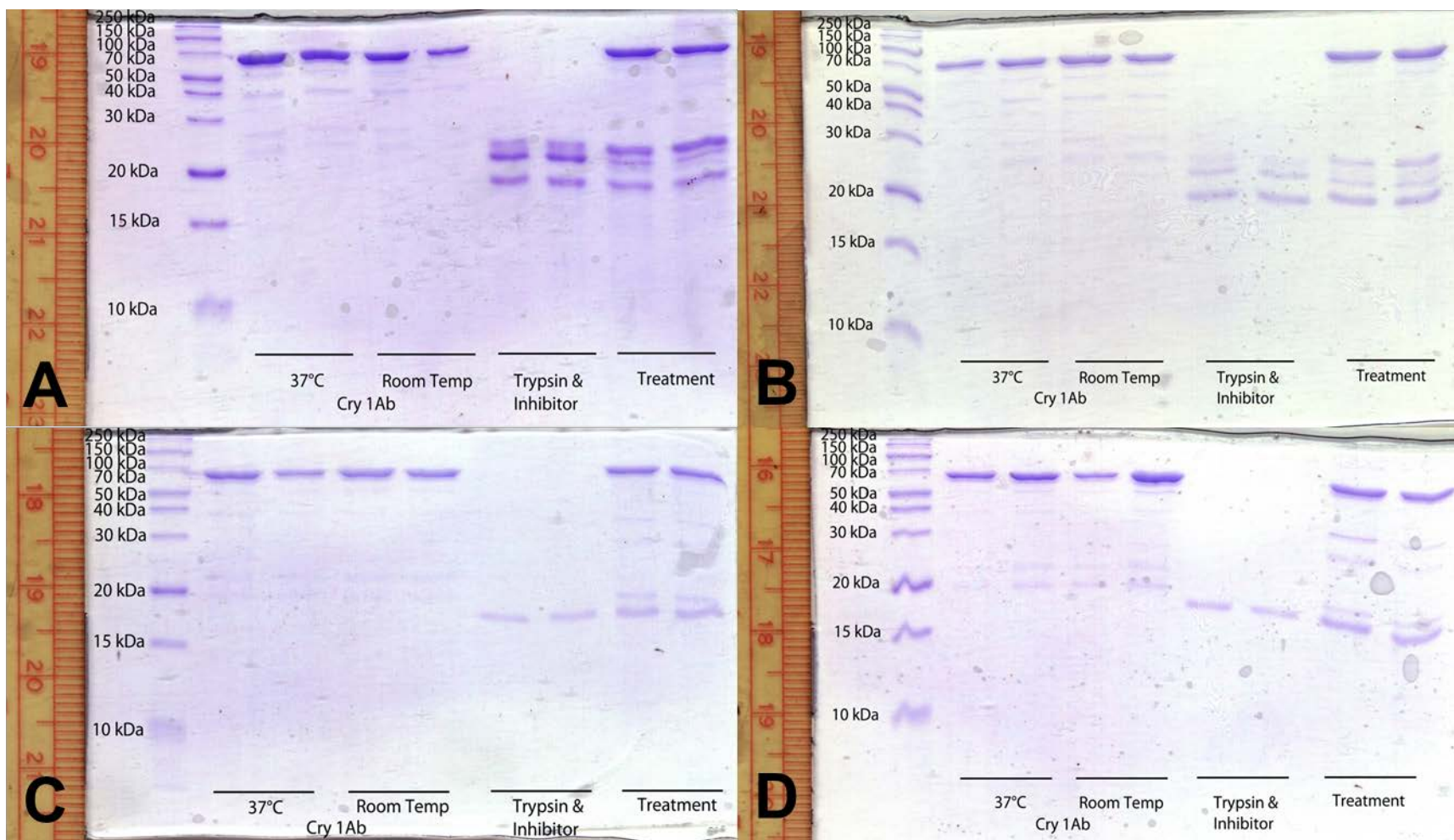




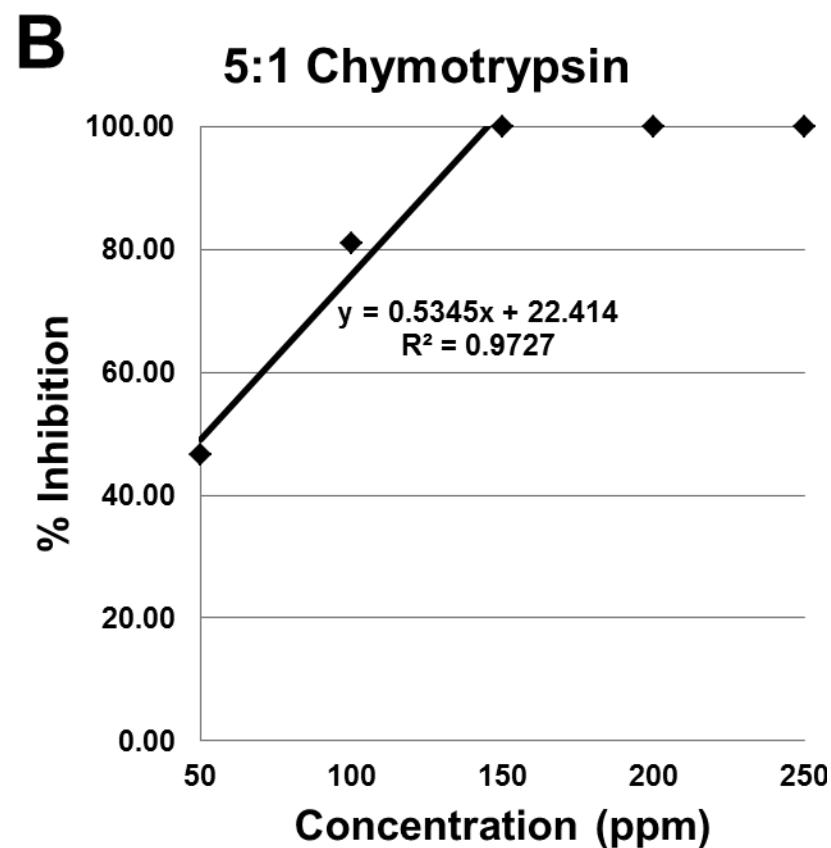
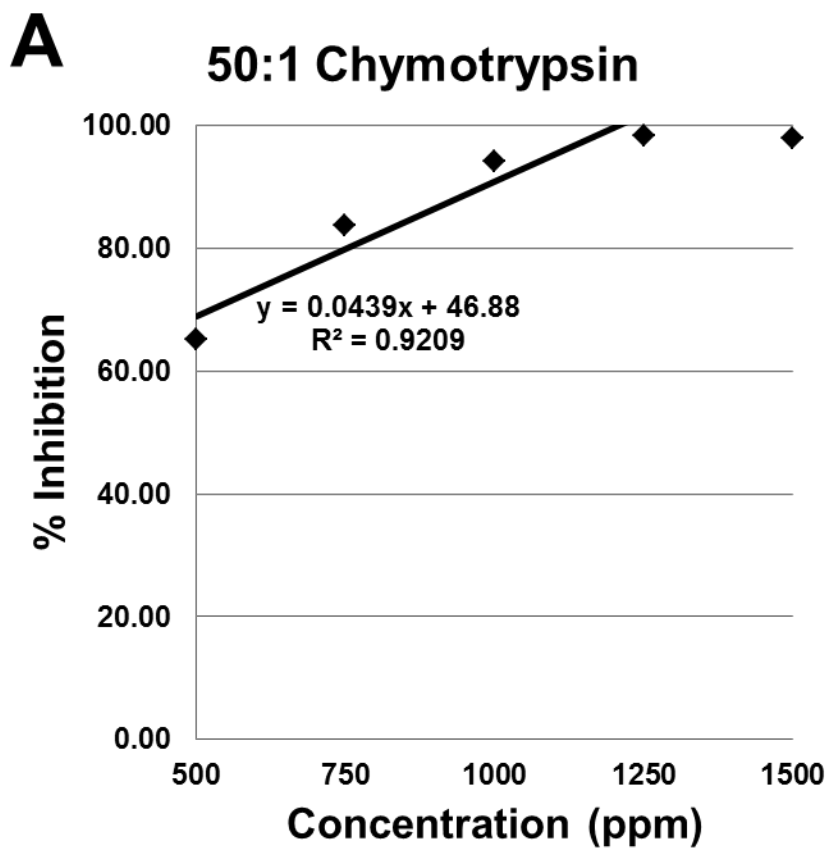
**Figure 3.** Trypsin activity assay results. Complete inhibition is achieved at 147  $\mu\text{g/mL}$  trypsin inhibitor.



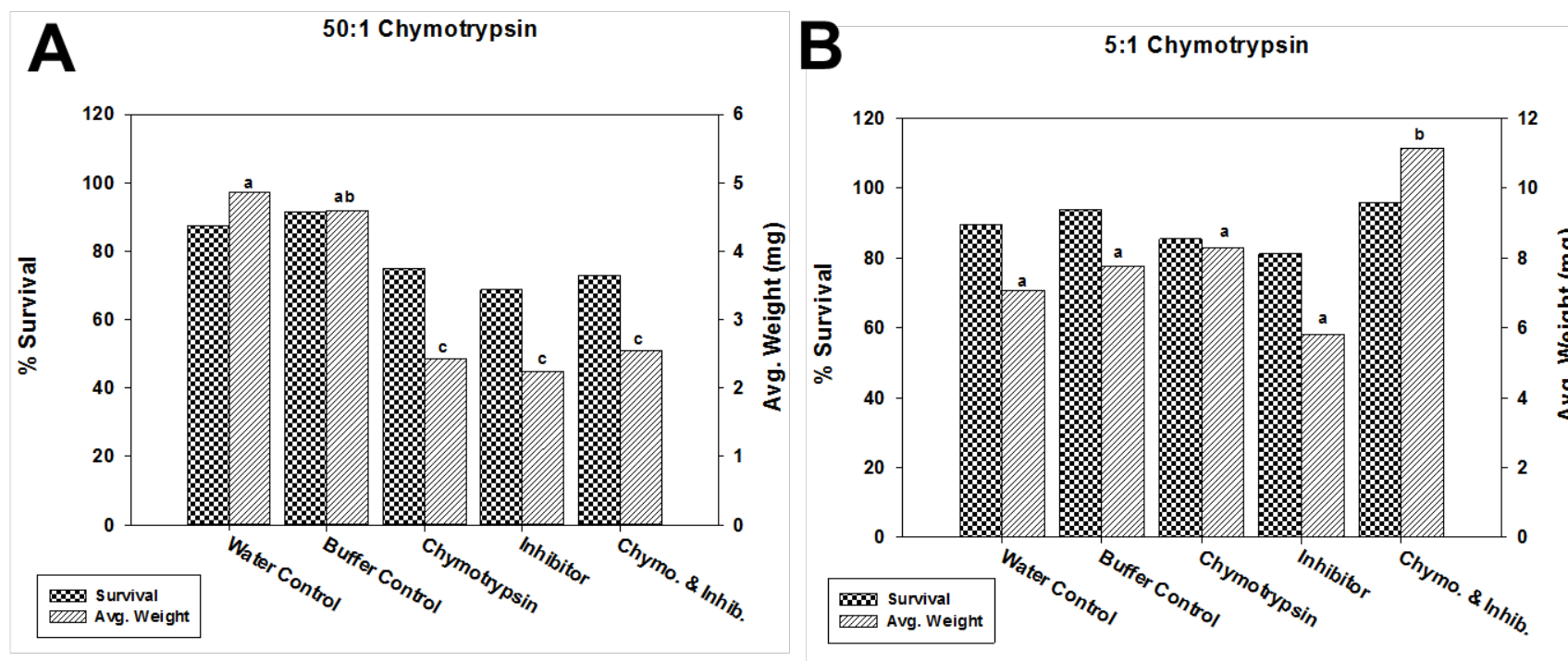
**Figure 4.** Bioassays with trypsin and its corresponding inhibitor, trypsin inhibitor from *Glycine max*. Uppercase letters indicate significance within survival across treatments ( $p = 0.05$ ). Lowercase letters indicate significance within average weight across treatments ( $p = 0.05$ ).



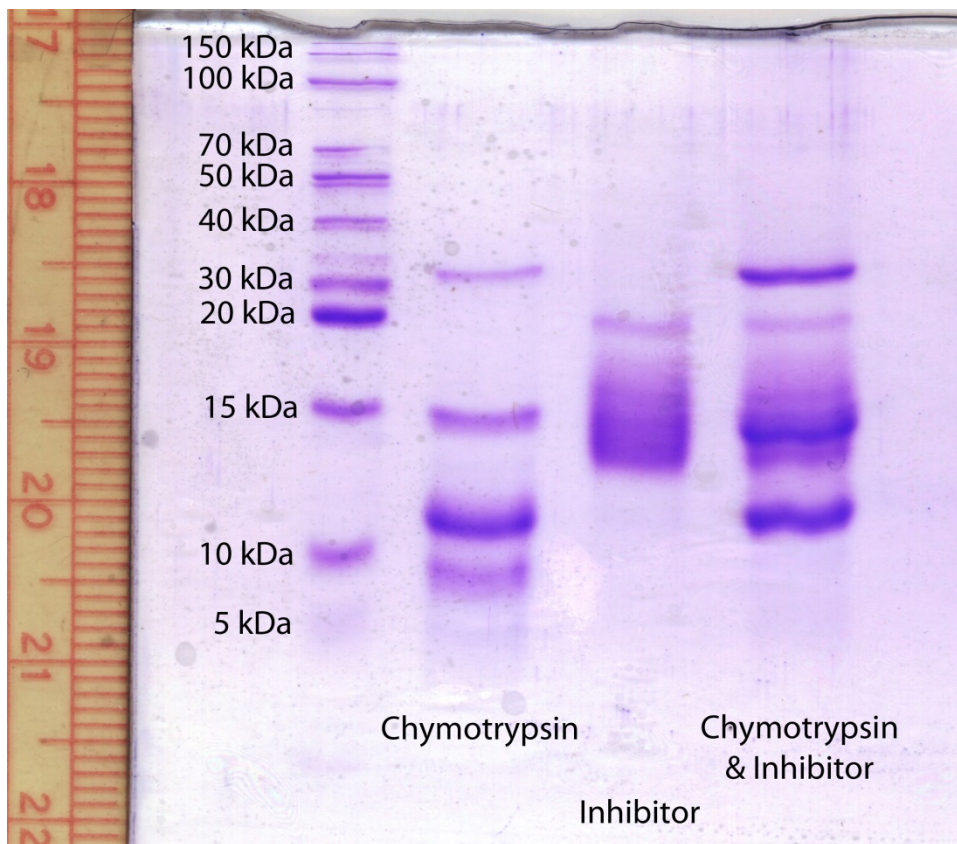
**Figure 5.** Degradation of Cry1Ab with the trypsin model system. Incubation times: **A** – 10 minutes, **B** – 1 hr, **C** – 8 hr, **D** – 24 hr.



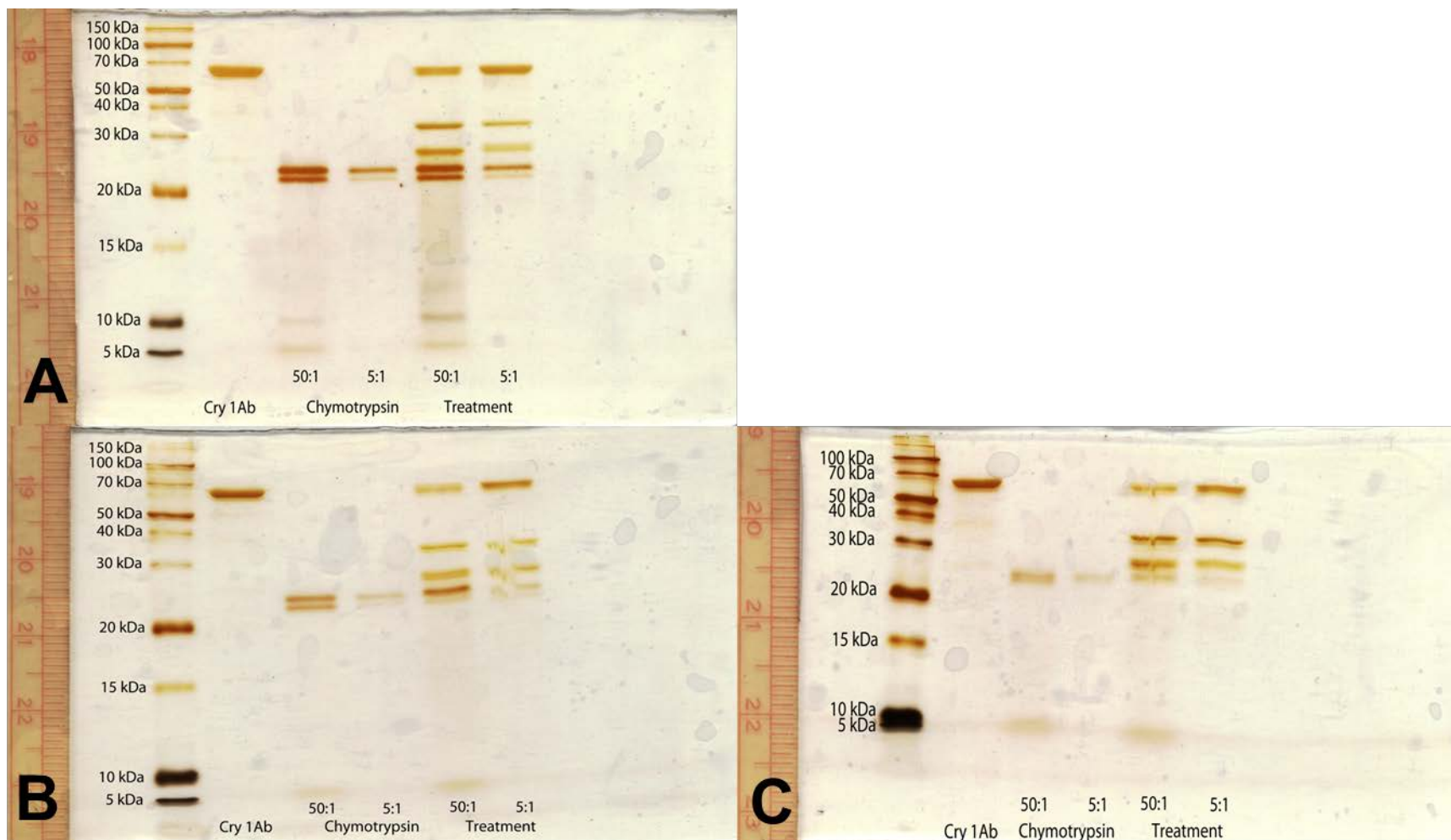
**Figure 6.** Chymotrypsin activity assay results. Complete inhibition is achieved at 1,210  $\mu\text{g/mL}$  of trypsin-chymotrypsin inhibitor (50:1 chymotrypsin) and 145  $\mu\text{g/mL}$  of trypsin-chymotrypsin inhibitor (5:1 chymotrypsin).



**Figure 7.** Bioassays with chymotrypsin and its corresponding inhibitor, trypsin-chymotrypsin inhibitor. **A** 50:1 chymotrypsin. **B** 5:1 chymotrypsin. There were no significant differences in survival for either concentration of chymotrypsin. Lowercase letters indicate significance within average weight across treatments ( $p = 0.05$ ).

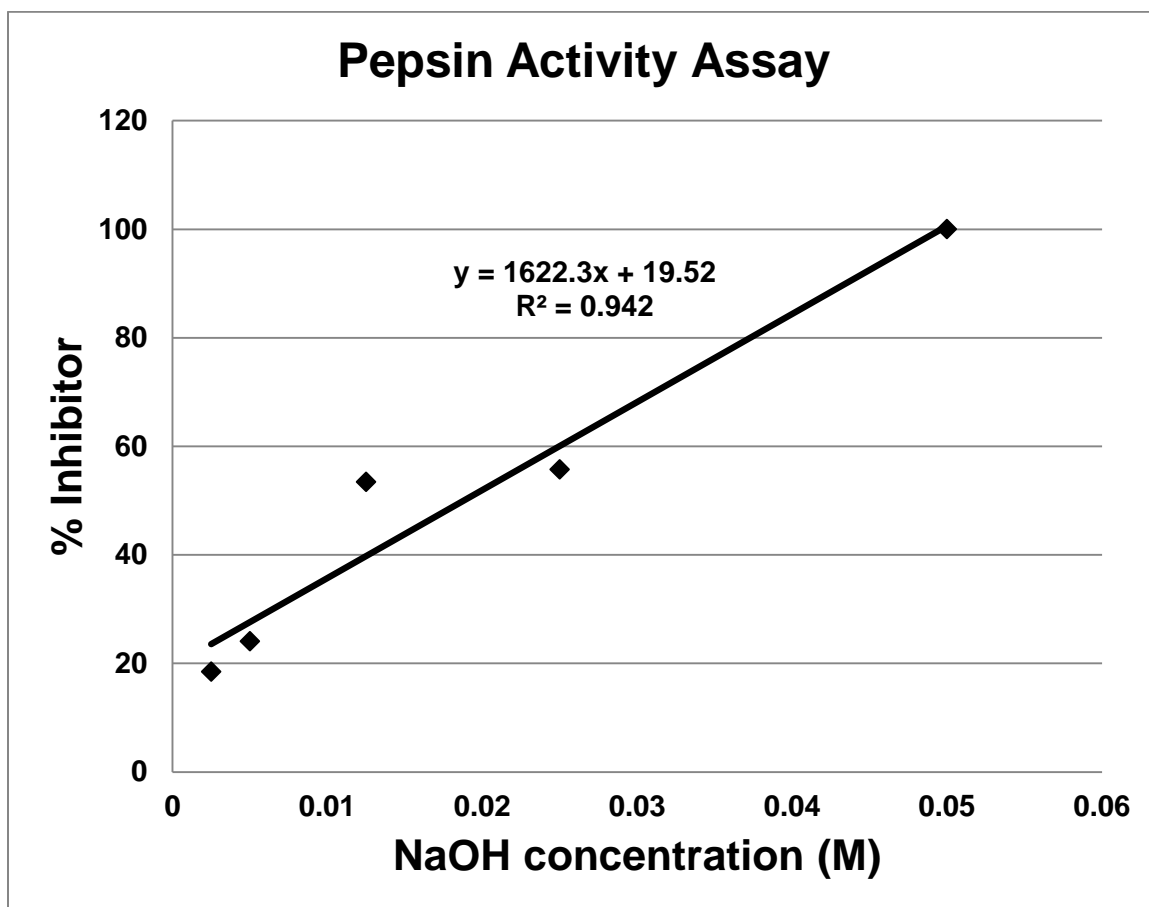


**Figure 8.** Chymotrypsin test gel to determine if visualization of Cry protein fragment bands would be impaired by chymotrypsin and its inhibitor. Numerous bands are present in the sample with chymotrypsin and its inhibitor, which could make identification of fragments difficult.



**Figure 9.** Degradation of Cry1Ab with the chymotrypsin model system. Incubation times: **A** – 1 hr, **B** – 8 hr, **C** – 24 hr. Degradation of the protein occurs at all three time points, with only two Cry1Ab fragment bands visualized.

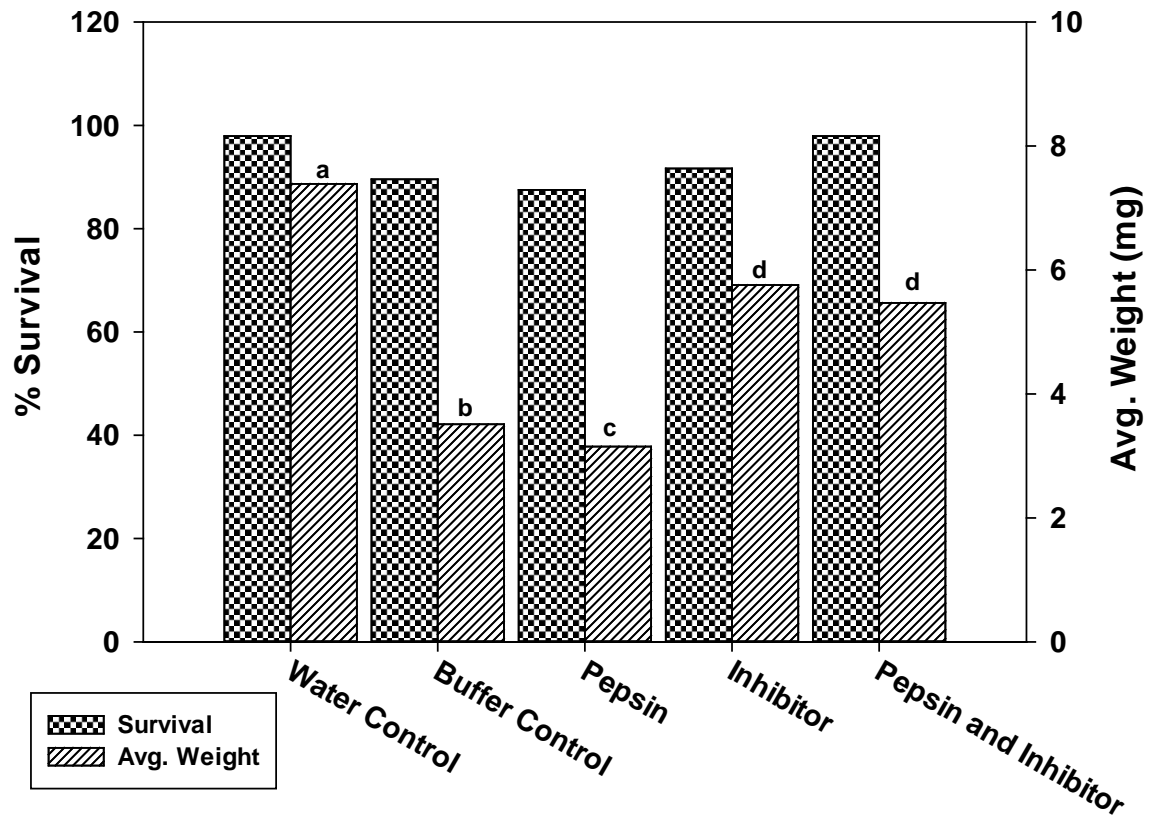




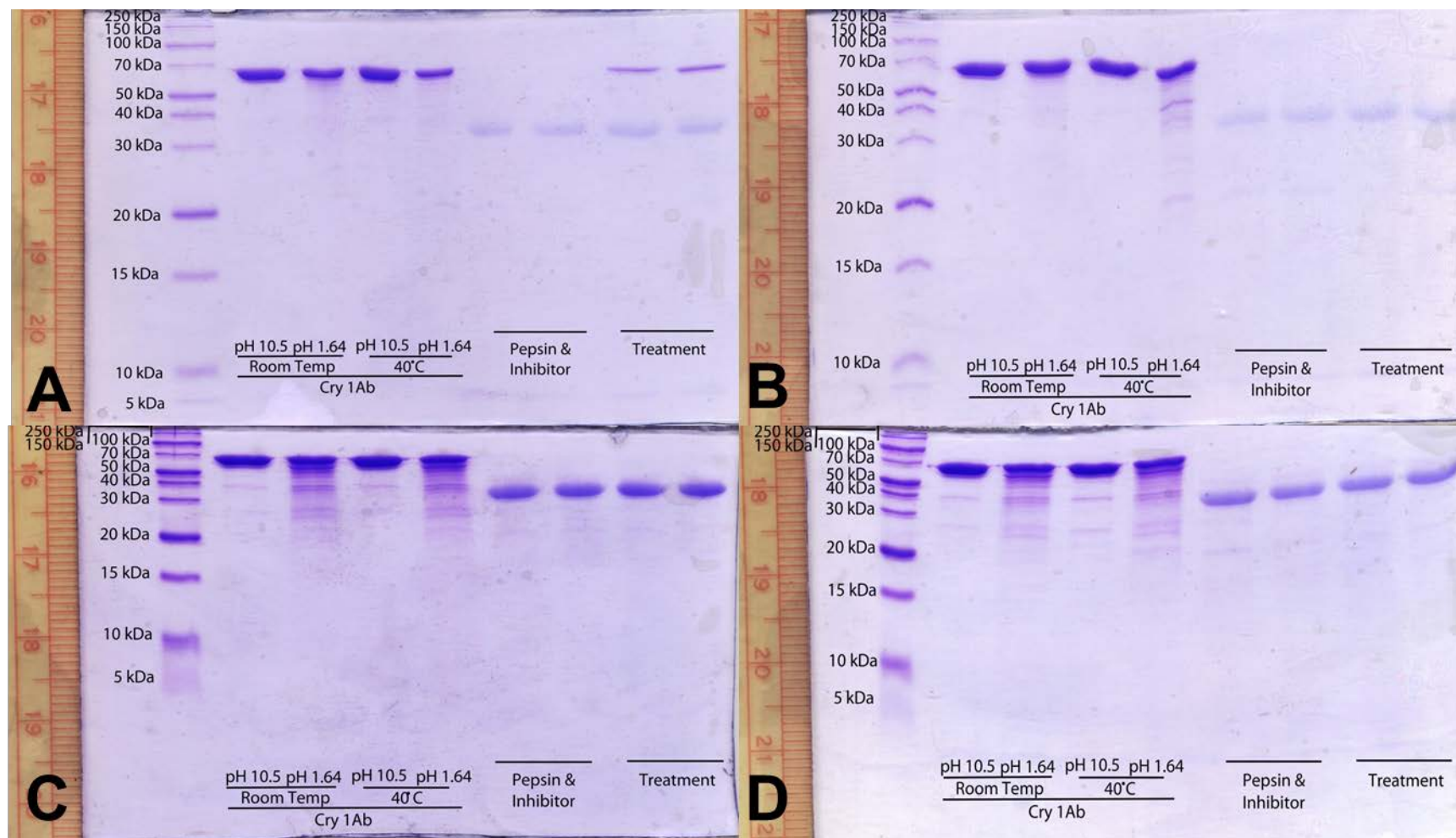
**Figure 10.** Pepsin activity assay results. Complete inhibition is achieved at 0.0496 M NaOH.



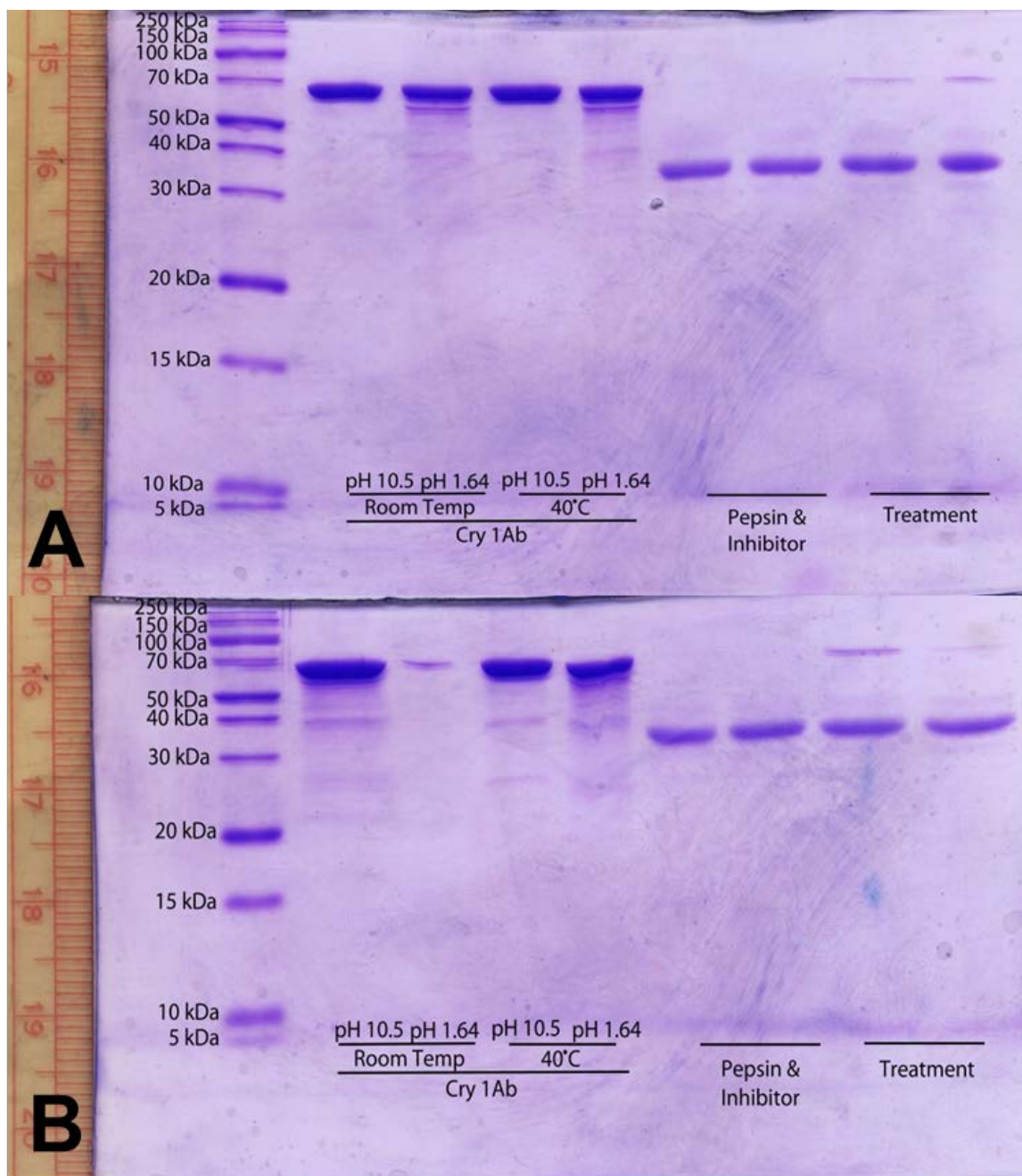
### Pepsin Bioassay



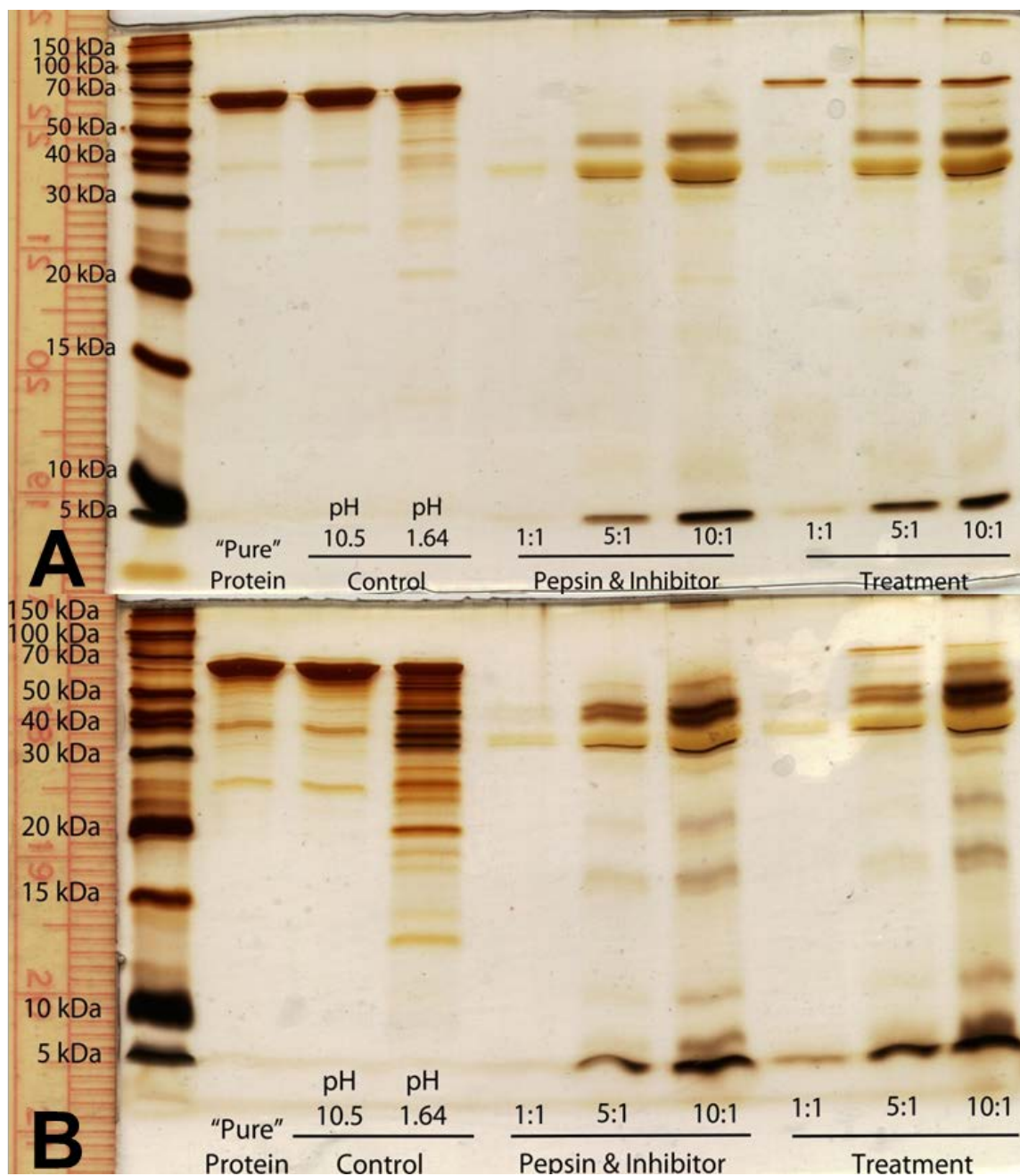
**Figure 11.** Bioassays with pepsin and its corresponding inhibitor, sodium hydroxide. Lowercase letters indicate significance within average weight across treatments ( $p = 0.05$ ).



**Figure 12.** Degradation of Cry1Ab with the 10:1 pepsin model system. Incubation times: **A** – 10 minutes, **B** – 30 minutes, **C** – 1 hr, **D** – 4 hr.

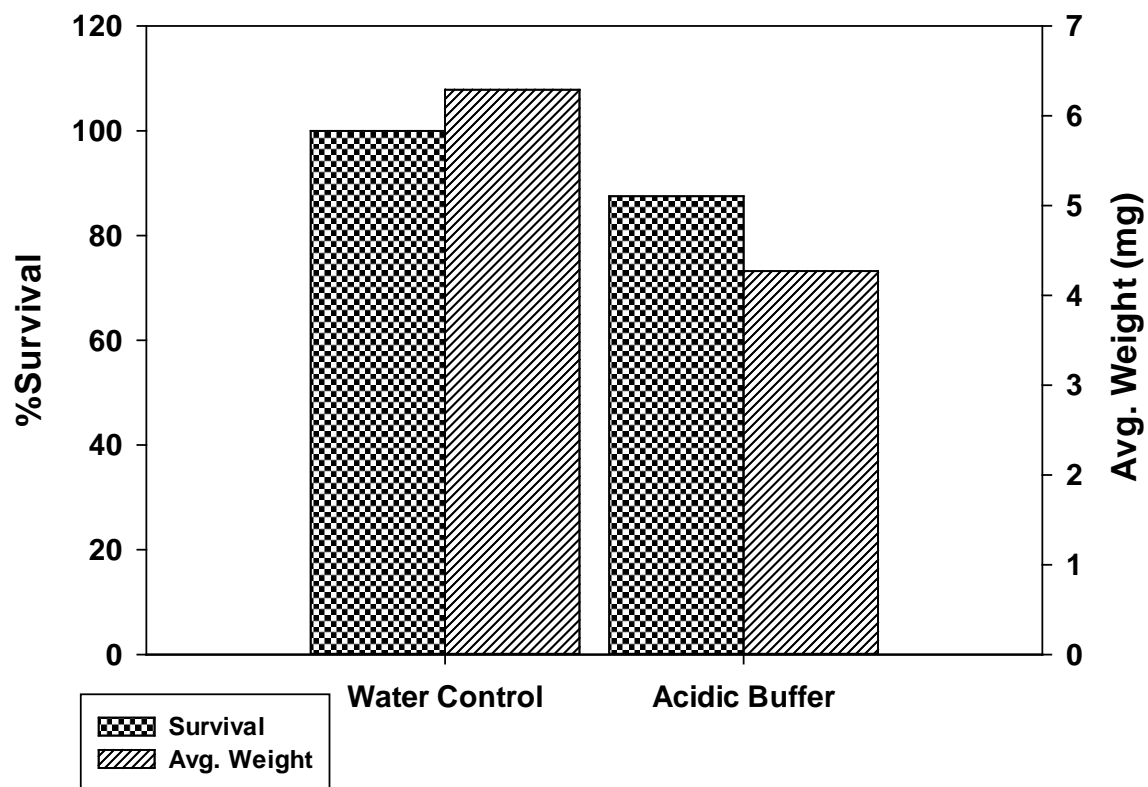


**Figure 13.** Degradation of Cry1Ab with the 5:1 pepsin model system. Incubation times: **A** – 1 hr, **B** – 4 hr.



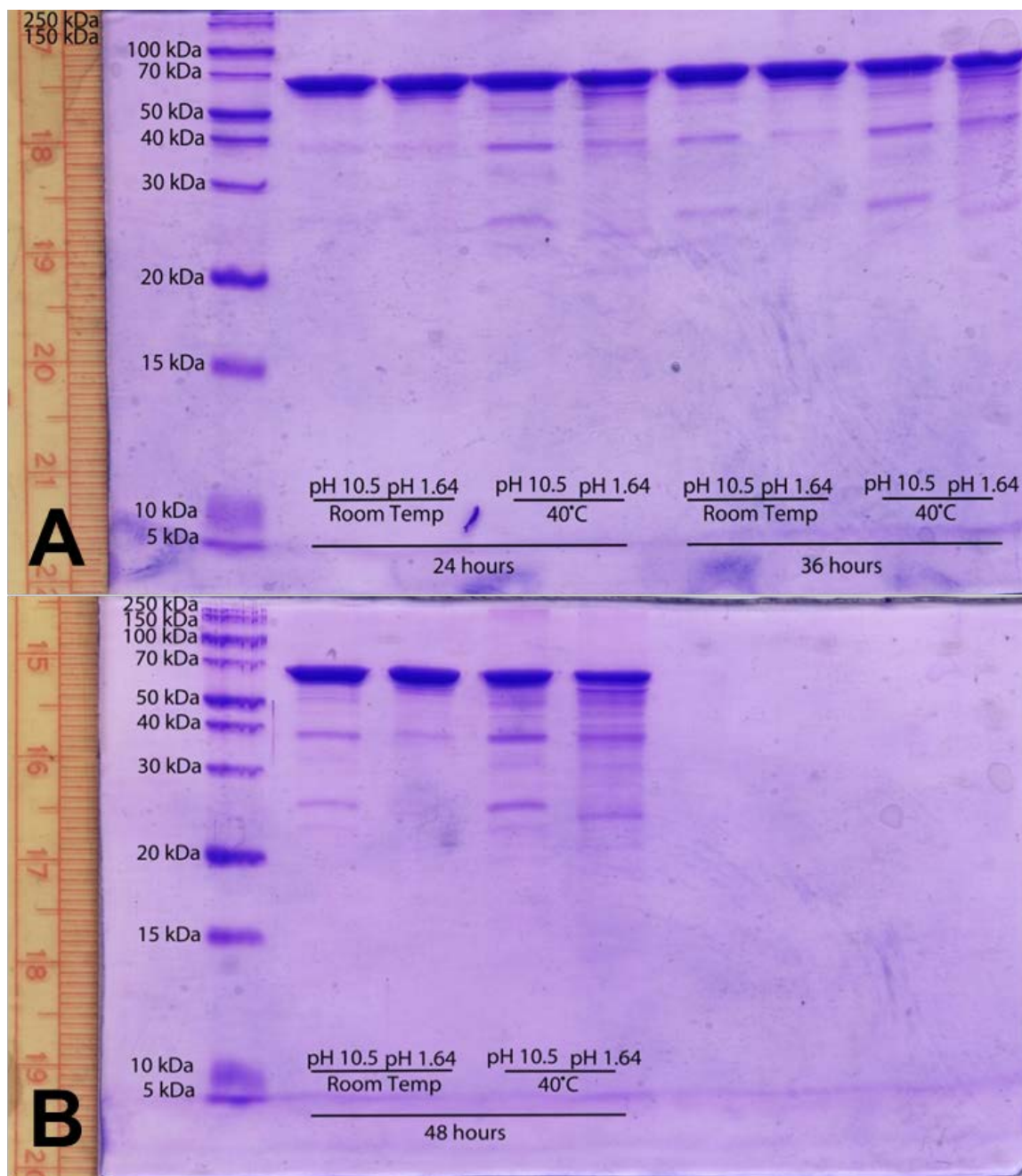
**Figure 14.** Degradation of Cry1Ab with the three pepsin model systems. Incubation times: **A** – 30 minutes, **B** – 1 hr.

### Acidic Buffer Bioassay

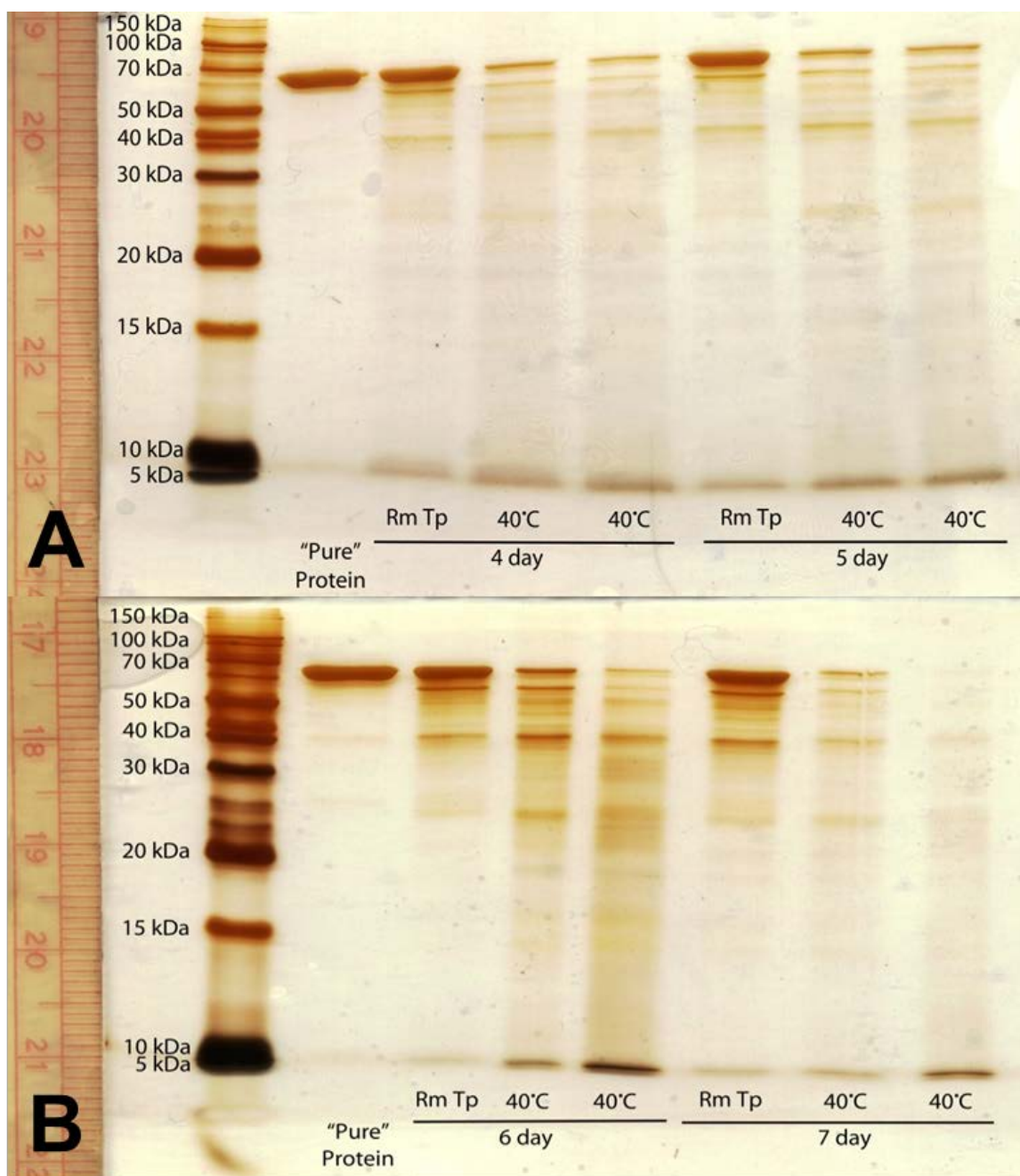


**Figure 15.** Bioassays with acidic buffer. No significant differences were observed for survival or average weight between the water control and the acidic buffer at  $p = 0.05$ .

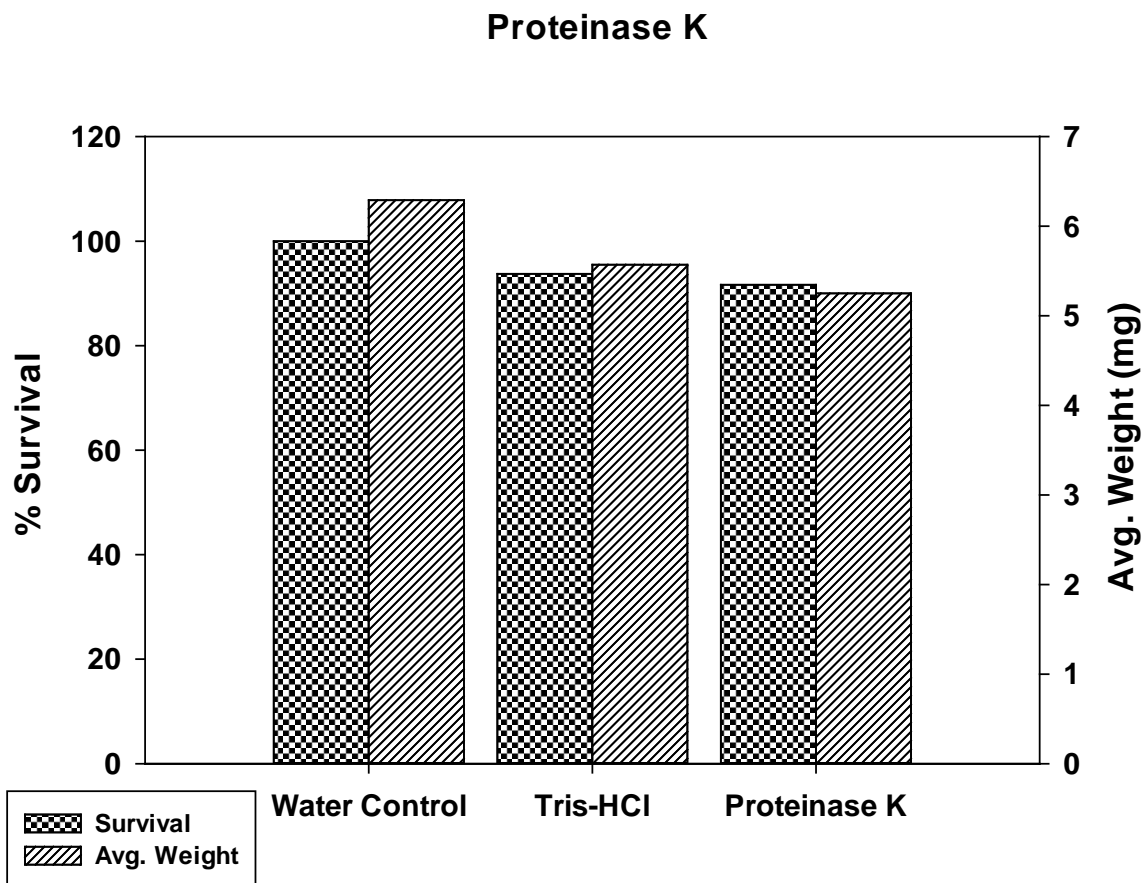




**Figure 16.** Degradation of Cry1Ab with the acidic buffer model system. Incubation times: **A** – 24 and 36 hr, **B** – 48hr.

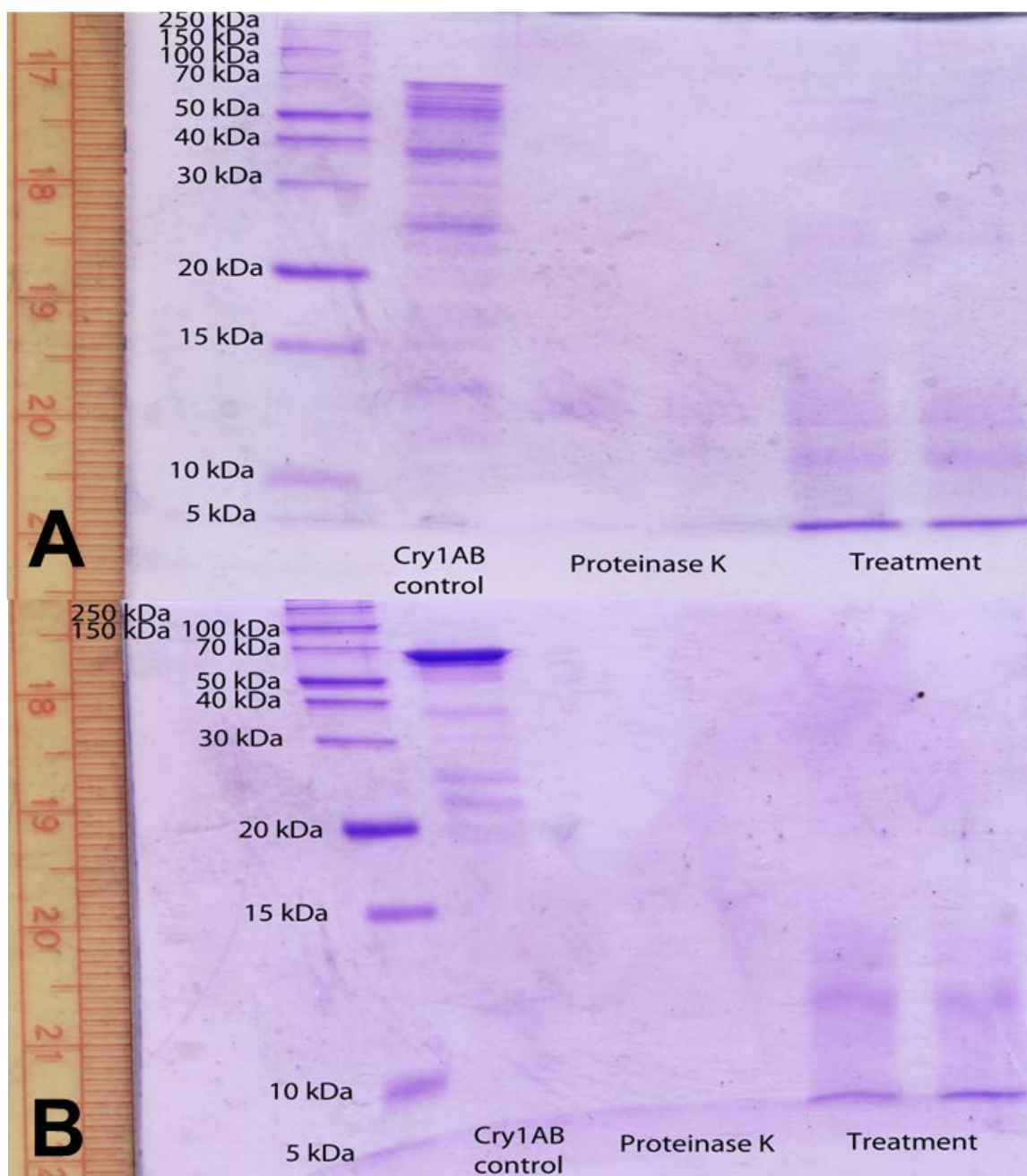


**Figure 17.** Degradation of Cry1Ab with the acidic buffer model system. Incubation times: **A** – 4-5 days, **B** – 6-7 days.

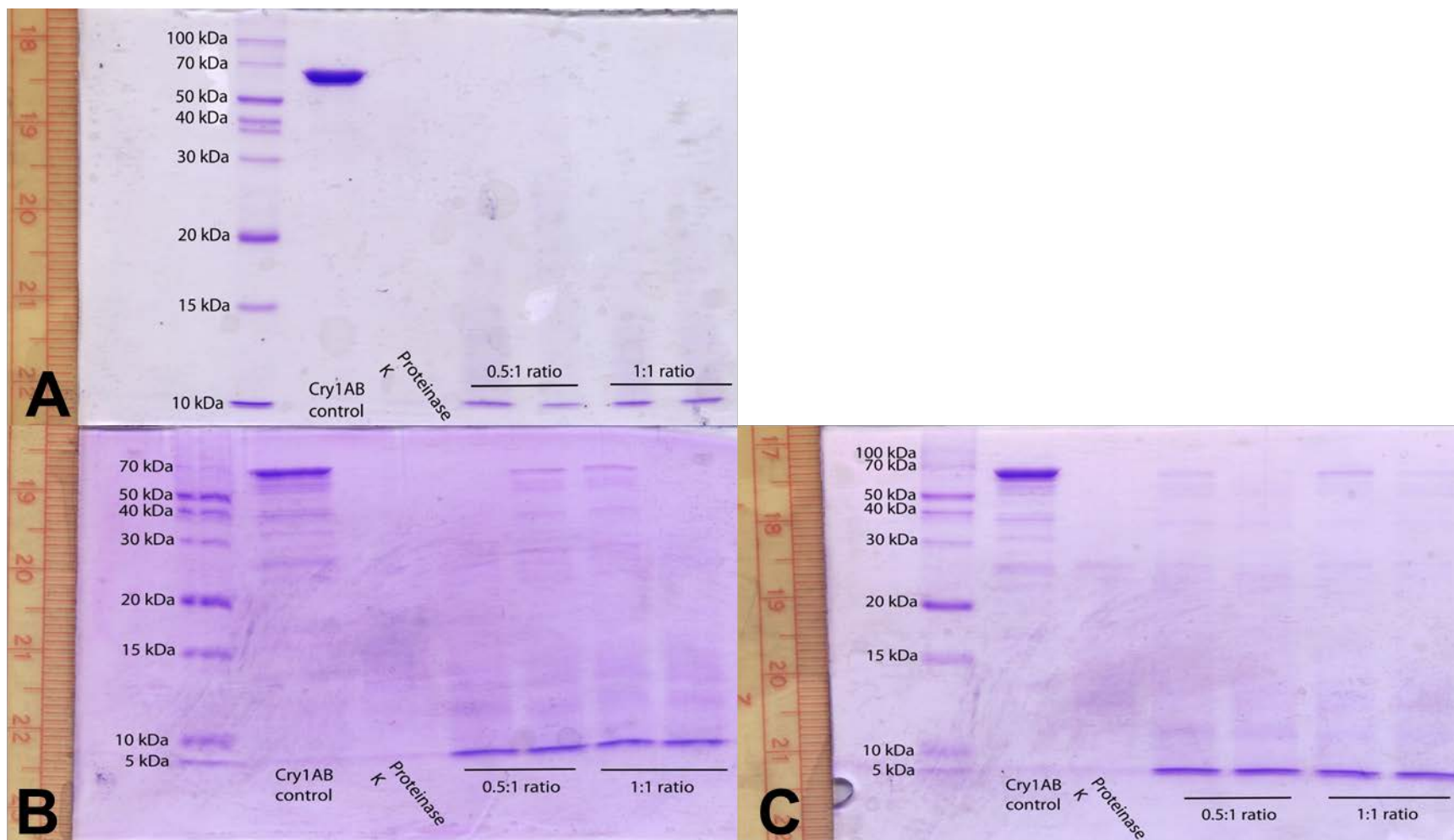


**Figure 18.** Bioassays with proteinase K. No significant differences were observed for survival or average weight between the water control and the acidic buffer at  $p = 0.05$ .

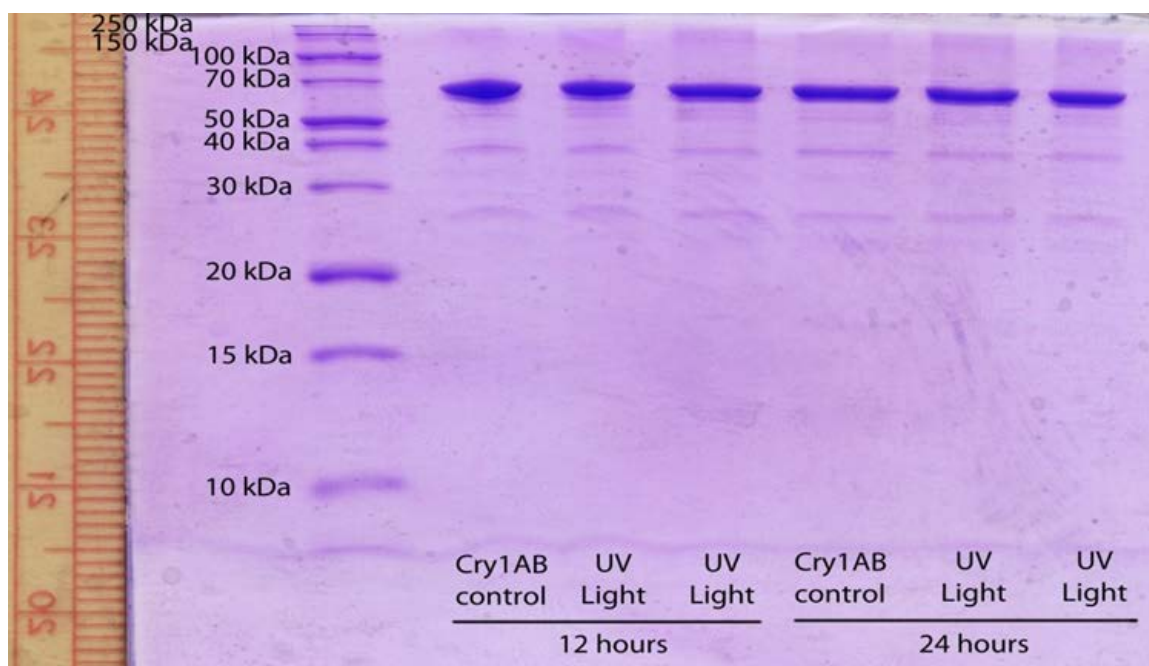




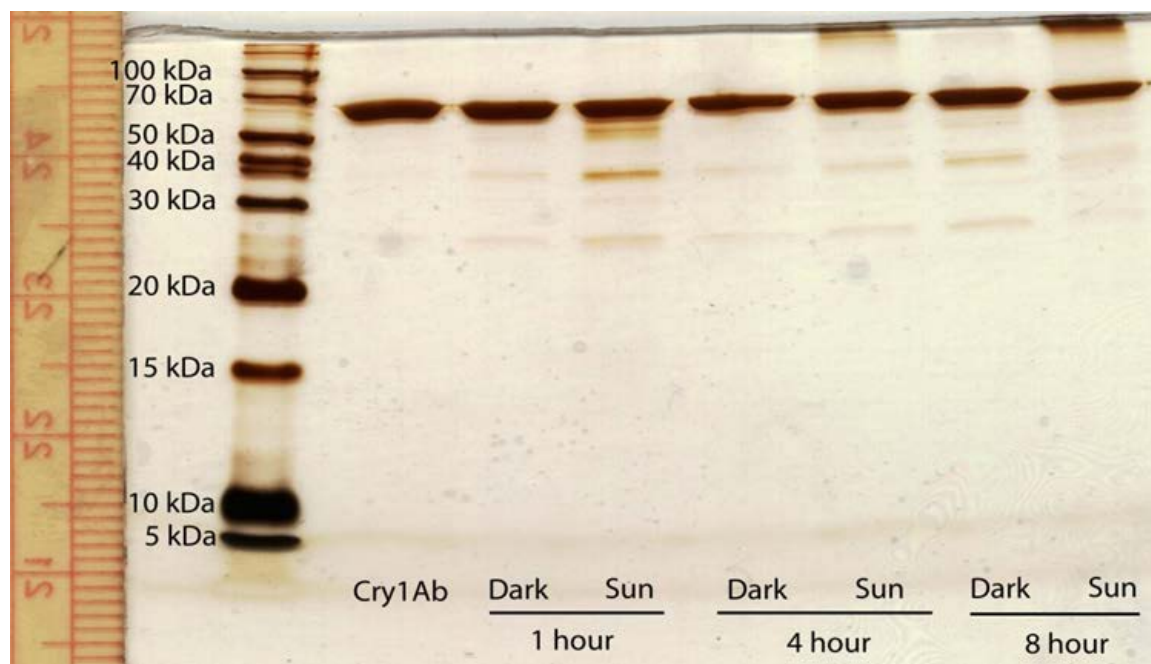
**Figure 19.** Degradation of Cry1Ab with the proteinase K model system. Incubation times: **A** – 1 hr, **B** – 24 hr.



**Figure 20.** Degradation of Cry1Ab with the proteinase K model system. Incubation times: **A** – 15 minutes, **B** – 30 minutes, **C** – 1 hr.

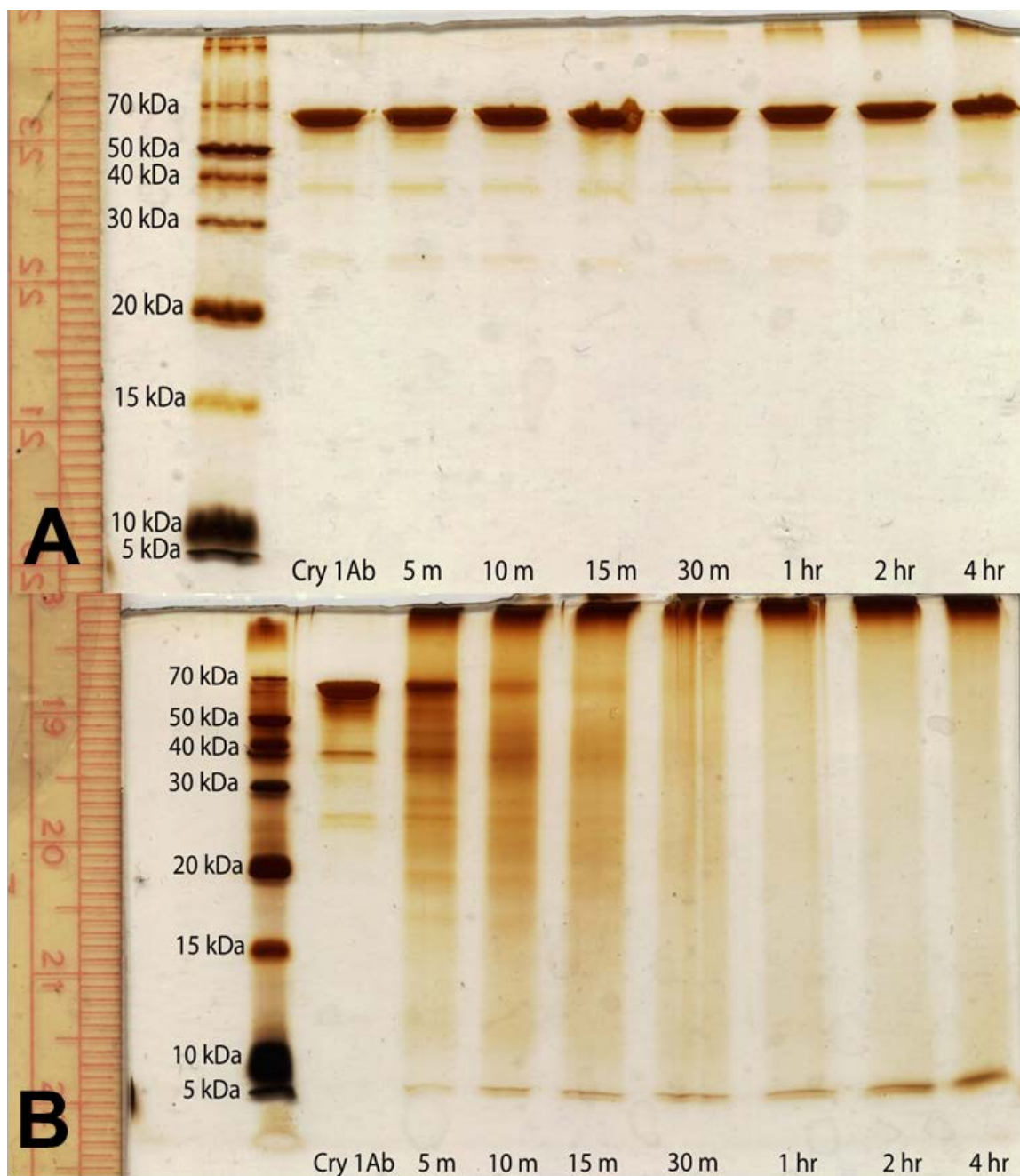


**Figure 21.** Degradation of Cry1Ab with the short wave UV model system.

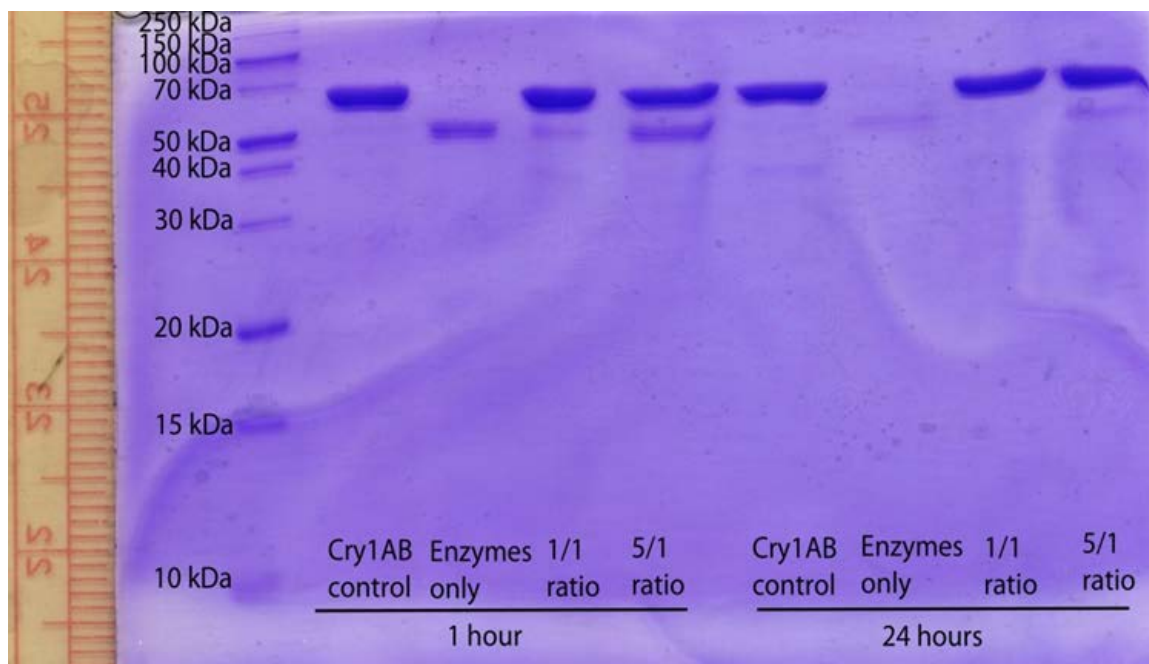


**Figure 22.** Degradation of Cry1Ab with the natural sunlight model system.

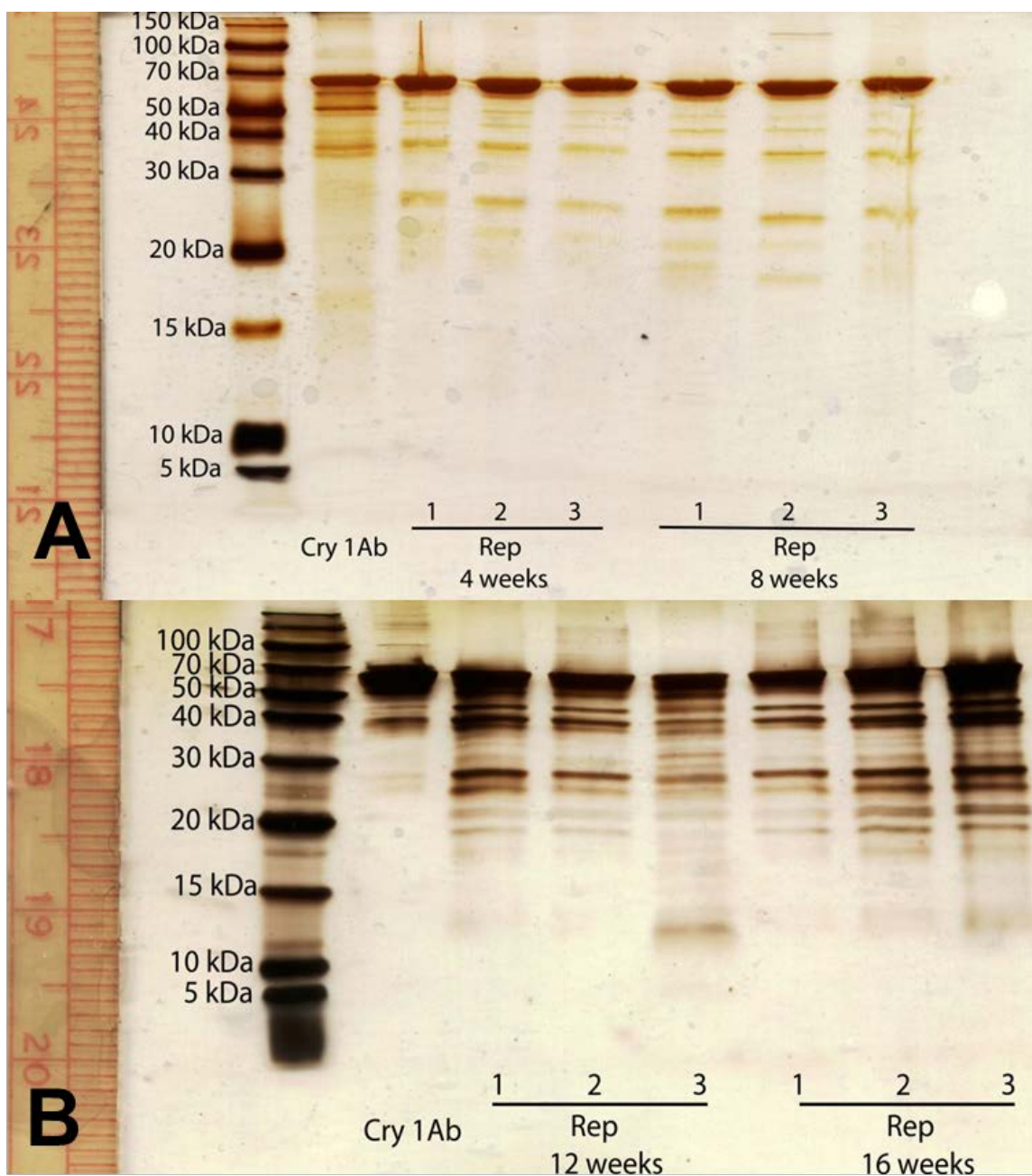




**Figure 23.** Degradation of Cry1Ab using a photoreactor. **A** – Degradation with 365 nm lamps, **B** – Degradation with 254 nm lamps.



**Figure 24.** Degradation of Cry1Ab with the simulated gut fluid model system.



**Figure 25.** Degradation of Cry1Ab in CAPS buffer over time. **A** – Degradation over 4 and 8 weeks, **B** – Degradation over 12 and 16 weeks.

## Tables

**Table 1.** Recipes for separating and stacking gel used in gel electrophoresis.

<b>Separating Gel</b>	<b>Stacking Gel</b>
2.474 mL 30% acrylamide	0.330 mL 30% acrylamide
1.847 mL 1M Tris-HCl, pH 8.8	0.247 mL 1M Tris-HCl, pH 6.8
0.610 mL nanopure water	1.391 mL nanopure water
50 $\mu$ L 10% (w/v) sodium dodecyl sulfate	20 $\mu$ L 10% (w/v) sodium dodecyl sulfate
4 $\mu$ L N,N,N',N'-tetramethylethylenediamine	2 $\mu$ L N,N,N',N'-tetramethylethylenediamine
17 $\mu$ L 10% (w/v) ammonium persulfate	10 $\mu$ L 10% (w/v) ammonium persulfate



**Table 2.** Buffer candidates tested to determine potential effects on European corn borer larvae.

Buffer	Composition	Enzyme compatibility	References
1	10 mM phosphate pH 7.0-8.0	Trypsin Chymotrypsin	Araujo <i>et al.</i> [28] Yin <i>et al.</i> [29]
2	1 mM Tris-HCl 1 M Urea 0.01% (w/v) SDS pH 8.5	Trypsin	Hitchcock <i>et al.</i> [30]
3	0.1 M Potassium phosphate pH 7.6	Trypsin Chymotrypsin Pepsin (pH 2.0)	Hamaker <i>et al.</i> [31]
4	98 mM Sodium bicarbonate 2 mM Sodium carbonate pH 8.0	Trypsin Chymotrypsin	Singh and Creamer [32]
5	0.1 M Tris-HCl 10 mM Calcium chloride pH 7.8	Trypsin Chymotrypsin	DeMar <i>et al.</i> [33] Nielsen <i>et al.</i> [34]
6	50 mM HCl	Pepsin	Nielsen <i>et al.</i> [34]
7	50 mM KCl 30 mM HCl pH 1.5	Pepsin	Goni <i>et al.</i> [35]

**Table 3.** Samples and procedure used in the trypsin activity assay.

<b>Reagent Blank</b>	<b>Standard</b>	<b>Sample Blank</b>	<b>Samples</b>
1 mL $\text{KH}_2\text{PO}_4$	1 mL $\text{KH}_2\text{PO}_4$ 1 mL trypsin	1 mL inhibitor	1 mL inhibitor 1 mL trypsin
<b>Heat at 37°C for 10 minutes</b>			
2.5 mL BAPA	2.5 mL BAPA	2.5 mL BAPA	2.5 mL BAPA
<b>Heat at 37°C for 10 minutes</b>			
0.5 mL 30% (v/v) acetic acid 1 mL trypsin	0.5 mL 30% (v/v) acetic acid	0.5 mL 30% (v/v) acetic acid 1 mL trypsin	0.5 mL 30% (v/v) acetic acid
<b>Filter with Whatman #3</b>			
<b>Measure at 410 nm</b>			

**Table 4.** Layout for trypsin model system. At the start of the experiment, the solutions in the initiation column were combined in a vial. All samples were held at 37°C for 10 min or 1, 8, or 24 hr. At the end of the experiment, the solution in the termination column was added. All samples were flash frozen in liquid nitrogen and stored at -80°C until analysis.

Samples	Initiation	Termination
Cry1Ab Control	75 $\mu\text{L}$ $\text{KH}_2\text{PO}_4$ 75 $\mu\text{L}$ Cry1Ab solution	150 $\mu\text{L}$ $\text{KH}_2\text{PO}_4$
Trypsin + Inhibitor control	75 $\mu\text{L}$ $\text{KH}_2\text{PO}_4$ 75 $\mu\text{L}$ 5:1 trypsin solution in $\text{KH}_2\text{PO}_4$	150 $\mu\text{L}$ 200 $\mu\text{g/mL}$ inhibitor solution
Treatment	75 $\mu\text{L}$ Cry1Ab solution 75 $\mu\text{L}$ 5:1 trypsin solution in $\text{KH}_2\text{PO}_4$	150 $\mu\text{L}$ 200 $\mu\text{g/mL}$ inhibitor solution

**Table 5.** Samples and procedure used in the chymotrypsin activity assay.

<b>Reagent Blank</b>	<b>Standard</b>	<b>Sample Blank</b>	<b>Samples</b>
0.5 mL $\text{KH}_2\text{PO}_4$	0.5 mL $\text{KH}_2\text{PO}_4$ 0.5 mL chymotrypsin	0.5 mL inhibitor	0.5 mL inhibitor 0.5 mL chymotrypsin
<b>Heat at 37°C for 10 minutes</b>			
2.5 mL GPANA	2.5 mL GPANA	2.5 mL GPANA	2.5 mL GPANA
<b>Heat at 37°C for 10 minutes</b>			
1 mL 30% (v/v) acetic acid 0.5 mL chymotrypsin	1 mL 30% (v/v) acetic acid	1 mL 30% (v/v) acetic acid 0.5 mL chymotrypsin	1 mL 30% (v/v) acetic acid
<b>Filter with Whatman #3</b>			
<b>Measure at 410 nm</b>			

**Table 6.** Layout for chymotrypsin model system. At the start of the experiment, the solutions in the initiation column were combined in a vial. Two levels of chymotrypsin were used: a 50:1 mol:mol chymotrypsin:toxin ratio and a 5:1 mol:mol ratio. All samples were held at 37°C for 1, 8, or 24 hr. At the end of the experiment, the solution in the termination column was added. All samples were flash frozen in liquid nitrogen and stored at -80°C until analysis.

Samples	Initiation	Termination
Cry1Ab Control	75 $\mu\text{L}$ $\text{KH}_2\text{PO}_4$ 75 $\mu\text{L}$ Cry1Ab solution	150 $\mu\text{L}$ water
Chymotrypsin control	75 $\mu\text{L}$ $\text{KH}_2\text{PO}_4$ 75 $\mu\text{L}$ chymotrypsin solution in $\text{KH}_2\text{PO}_4$	150 $\mu\text{L}$ water
Treatment	75 $\mu\text{L}$ Cry1Ab solution 75 $\mu\text{L}$ chymotrypsin solution in $\text{KH}_2\text{PO}_4$	150 $\mu\text{L}$ water

**Table 7.** Samples and procedure used in the pepsin activity assay.

<b>Reagent Blank</b>	<b>Standard</b>	<b>Sample Blank</b>	<b>Samples</b>
0.5 mL pepsin 0.5 mL buffer 0.5 mL 10% (w/v) NaHCO <sub>3</sub>	0.5 mL pepsin 0.5 mL buffer	0.5 mL NaOH	0.5 mL pepsin 0.5 mL NaOH
<b>Heat at 37°C for 10 minutes</b>			
0.5 mL succinyl-albumin	0.5 mL succinyl-albumin	0.5 mL succinyl-albumin	0.5 mL succinyl-albumin
<b>Heat at 37°C for 30 minutes</b>			
0.5 mL 10 mM TNBS	0.5 mL 10% (w/v) NaHCO <sub>3</sub> 0.5 mL 10 mM TNBS	0.5 mL 10% (w/v) NaHCO <sub>3</sub> 0.5 mL 10 mM TNBS	0.5 mL 10% (w/v) NaHCO <sub>3</sub> 0.5 mL 10 mM TNBS
<b>Heat at 50°C for 10 minutes</b>			
<b>Measure at 490 nm</b>			

**Table 8.** Layout for pepsin model system. At the start of the experiment, the solutions in the initiation column were combined in a vial. Two levels of pepsin were used: a 50:1 mol:mol pepsin:toxin ratio and a 5:1 mol:mol ratio. One basic and one acidic buffer control were held at room temperature, all other controls and samples were held at 40°C. Incubation times: 10:1 mol:mol ratio – 10 and 30 min, 1 and 4 hr; 5:1 mol:mol ratio – 1 and 4 hr. At the end of the experiment, the solution in the termination column was added. All samples were flash frozen in liquid nitrogen and stored at -80°C until analysis.

Samples	Initiation	Termination
Cry1Ab Buffer Control (pH 10.5) Room Temp.	75 µL CAPS 75 µL Cry1Ab solution	150 µL water
Cry1Ab Buffer Control (pH 1.64) Room Temp.	75 µL $\text{KH}_2\text{PO}_4$ 75 µL Cry1Ab solution	150 µL water
Cry1Ab Buffer Control (pH 10.5) - 40°C	75 µL CAPS 75 µL Cry1Ab solution	150 µL water
Cry1Ab Buffer Control (pH 1.64) - 40°C	75 µL $\text{KH}_2\text{PO}_4$ 75 µL Cry1Ab solution	150 µL water
Pepsin & Inhibitor Control	75 µL Pepsin 75 µL CAPS	150 µL NaOH
Treatment	75 µL Pepsin 75 µL Cry1Ab solution	150 µL NaOH

**Table 9.** Layout for proteinase K model system. At the start of the experiment, the solutions in the initiation column were combined in a vial. Two levels of proteinase K were used in the treatment: a 1:1 mol:mol proteinase K:toxin ratio and a 0.5:1 mol:mol ratio. All samples were held at 37°C for 1 and 24 hr. At the end of the experiment, the solution in the termination column was added. All samples were flash frozen in liquid nitrogen and stored at -80°C until analysis.

Samples	Initiation	Termination
Cry1Ab Control	75 µL Tris-HCl 75 µL Cry1Ab solution	150 µL water
Proteinase K Control	75 µL CAPS 75 µL proteinase K solution in Tris-HCl	150 µL water
Treatment	75 µL Cry1Ab solution 75 µL proteinase K solution in Tris-HCl	150 µL water



**Table 10.** Layout for simulated gut fluid model system. At the start of the experiment, the solutions in the initiation column were combined in a vial. Two levels of enzymes were used in the treatment: a 5:1 mol:mol enzyme:toxin ratio and a 1:1 mol:mol ratio. All samples were held at 40°C for 1 and 24 hr. At the end of the experiment, the solution in the termination column was added. All samples were flash frozen in liquid nitrogen and stored at -80°C until analysis.

Samples	Initiation	Termination
Cry1Ab Control	75 $\mu$ L $\text{KH}_2\text{PO}_4$ 75 $\mu$ L Cry1Ab solution	150 $\mu$ L water
Enzyme Control	75 $\mu$ L CAPS 75 $\mu$ L 5:1 enzyme solution	150 $\mu$ L water
1:1 Treatment	75 $\mu$ L Cry1Ab solution 75 $\mu$ L 1:1 enzyme solution	150 $\mu$ L water
5:1 Treatment	75 $\mu$ L Cry1Ab solution 75 $\mu$ L 5:1 enzyme solution	150 $\mu$ L water

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## **CHAPTER 3. ELISA DETECTION AND BIOACTIVITY OF CRY1AB PROTEIN FRAGMENTS**

*A paper to be submitted to Chemosphere*

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### **Abstract**

The continuing use of transgenic crops has led to an increased interest in the fate of Cry proteins in the environment. ELISAs have emerged as the preferred detection method for Cry proteins in environmental matrices. Concerns exist that ELISAs are capable of detecting fragments of Cry proteins, which may lead to an over-estimation of the concentration of these proteins in the environment. Five model systems were used to generate fragments of the Cry1Ab protein, which were then analyzed by ELISAs and bioassays. The fragments from four of the model systems were not detectable by ELISA and did not retain bioactivity. Fragments from the proteinase K model system were detectable by ELISA and retained bioactivity; the reason for this is not yet known. Despite this result, ELISAs appear to provide an accurate estimation of the amount of Cry proteins in the environment, as detectable fragments retained bioactivity, and non-detectable fragments did not retain bioactivity.

### **Introduction**

Transgenic maize expressing one or more insecticidal crystalline (Cry) proteins for management of agricultural pests now accounts for 81% of all maize planted in the United States (NASS, 2015). The widespread use of these Cry proteins has raised questions regarding the fate of the proteins in various environmental matrices. Numerous

researchers have attempted to address the questions of persistence, movement, stability, etc., by detecting and quantifying the amount of Cry proteins in various environmental matrices. Several methods of detections are available to monitor for Cry proteins in environmental samples, including Western blotting and bioassays; however, enzyme-linked immunosorbent assays (ELISA) have emerged as the preferred detection method (Brown 2011). ELISAs are a cost-effective method for researchers in academia and industry as the kits typically include all required materials and little specialized training is needed to analyze a large number of samples in a short time span (4-8 hours).

Most commercial ELISA kits are primarily designed and used for quality assurance/quality control procedures, i.e. the detection of Cry proteins in seed and plant tissues to distinguish genetically modified crops from non- genetically modified crops. Thus, before these ELISA kits can be adapted for use with environmental matrices, the analytical method, including extraction protocol, must be validated. The steps necessary for validation of ELISAs for use with environmental samples have been extensively studied and summarized elsewhere (Lipton *et al.* 2000, Grothaus *et al.* 2006, Schmidt and Alarcon 2011). The main steps are briefly summarized here: 1) sensitivity – determining the quantitative range and limit of detection and defining the upper and lower limit of quantitation; 2) specificity – determining there is no cross-reactivity or interference with similar proteins or matrix components to ensure only the protein of interest is detected; 3) accuracy – confirming the amount of protein detected in the ELISA is close to the actual amount in an environmental sample; 4) precision – minimizing variability to verify ELISA results are repeatable across days, analysts, and laboratories.

Biological validation of ELISAs to ensure the proteins detected in environmental samples retain bioactivity, is one area of validation that is understudied. A lack of biological validation is a major omission because most of the commercially available ELISA kits utilize polyclonal antibodies. Although the use of polyclonal antibodies leads to very sensitive assays because they bind to multiple epitopes on a protein and increase the chances of detection, their use also increases the likelihood of detecting a partially degraded protein, producing a false-positive result. Thus, even though some antibody-binding sites on a partially degraded protein may be lost, other epitopes on the protein fragment may be intact and capable of binding with the antibodies. The binding of antibodies to protein fragments could result in an ELISA test indicating a positive detection in a sample where no fully intact protein exists. The risk assessments for these proteins and their associated products could be impacted if the false positive results lead to overestimations of the concentrations of these proteins in the environment. For example, if ELISA results indicate the protein concentrations in the environment exceed the concentrations needed to cause adverse effects, then additional restrictions may be placed on the use of these products. Alternatively, if risk assessors use ELISA results that over-estimate the concentrations of intact Cry proteins in exposure assessments, then the risk assessments will be conservatively protective.

There is a basis for the concern that Cry protein fragments could produce false positive results. Einspanier *et al.* (2004) tracked Cry1Ab with a commercially available ELISA kit as the protein passed through the gastrointestinal tract (GIT) of cattle fed transgenic maize. Their results indicated that the protein accumulated within the GIT; this effect was not observed in cattle fed isoline, non-transgenic maize, so the authors ruled

out cross-reactivity with other proteins as the cause of the reported protein accumulation (Einspanier *et al.* 2004). A follow-up study tested the hypothesis that the protein was fragmented, yet still immunoreactive, leading to detection of the fragments with ELISA (Lutz *et al.* 2005). The authors fed transgenic maize containing Cry1Ab to cattle and collected samples from the GIT at slaughter. The samples were then analyzed with ELISAs and Western blotting. The ELISA results indicated that the Cry1Ab protein accumulated in the GIT, as reported in the first study. However, Western blotting only detected bands at approximately 17 and 34 kilodaltons (kDa); no fully intact Cry1Ab was detected, which contradicts the ELISA results (Lutz *et al.* 2005). Based on these results, understanding how fragmented proteins affect ELISAs and determining if any bioactivity is retained by these fragments is hugely important.

Five model systems were used to generate Cry1Ab fragments: chymotrypsin model system, proteinase K model system, acidic buffer model system, photodegradation model system, and long-term degradation in buffer model system. The solutions of fragments generated by these model systems will be analyzed with ELISAs to determine if the fragments are still detectable and analyzed by bioassays to determine if the fragments retain bioactivity.

## **Methods and Materials**

### *Gel Electrophoresis*

Fragmentation of the Cry1Ab protein was confirmed for each model system by polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE). Tris-HCl gels (15% acrylamide) used for resolution of larger fragments and parent protein (10-70 kDa) were purchased from Bio-Rad Laboratories Inc. (Hercules, CA, cat. No. 161-1157)



or were prepared fresh daily according to a previously described method (Rosenberg 1996). The recipes from Rosenberg (1996) were modified to fit a Bio-Rad Mini-Protean II gel apparatus (gel size 83 mm x 73 mm x 1 mm)(Table 1). An appropriate amount of 4X sample buffer was added to each sample to obtain a 1X sample buffer concentration. Sample buffer consisted 4 g sucrose, 0.8 g sodium dodecyl sulfate (SDS), 2.5 mL 1M Tris-HCl (pH 6.8) and 250  $\mu$ L 0.1% (w/v) bromophenol blue dissolved to 10 mL with nanopure water. Samples were boiled at 100°C for 3-4 min, chilled in ice for 10 min and centrifuged at 12,000 x g for 5 min. Running buffer (3.03 g Tris (hydroxymethyl)aminomethane (THAM), 14.42 g glycine, 1 g SDS, dilute to 1 L with nanopure water) was added to the upper and lower chambers of the apparatus. One well on each gels was loaded with 5  $\mu$ L of PageRuler™ Unstained Broad Range Protein Ladder (Fisher Scientific) with molecular bands ranging from 5 kilodaltons (kDa) to 250 kDa and 20  $\mu$ L of sample was added to the remaining wells. A constant 180 volts was applied to the gels until the dye front was within 1 centimeter of the bottom of the gel (approximate run times were 1 hour). Gels were stained with either Coomassie Brilliant Blue or silver stain and digitized using a Lexmark X83 All-in-One printer/copier/scanner and Lexmark Viewing Booth software (Lexmark International Inc., Lexington, KY). Adobe Photoshop CS5 (version 12.1 x64)(Adobe Systems Inc., San Jose, CA) was used to edit and annotate each gel.

### *Coomassie Staining*

The Coomassie staining procedure was adapted from Rosenberg (1996). Briefly, 1 g Coomassie Brilliant Blue R-250 (Fisher Scientific) was dissolved in 100 mL acetic acid and 400 mL methanol and diluted to 1 L with nanopure water. Destain solution was

prepared by diluting 100 mL acetic acid and 400 mL methanol to 1 L with nanopure water. Staining occurred for 30 min and was followed by destaining for 2-3 hours or overnight with the destain solution being changed 1-2 times. All staining occurred on an orbital shaker (ELMI Ltd., Riga, Latvia) at 100 rpm.

### *Silver Staining*

The silver staining procedure adapted from Rosenberg (1996) was used. Gels were stored at 4°C in gel fix solution (50% (v/v) methanol, 12% (v/v) acetic acid, 38% (v/v) water) overnight following gel electrophoresis. Each gel was washed three times with 50% (v/v) ethanol for 20 min each. Each gel was submerged in sodium thiosulfate (0.2 g/L sodium thiosulfate) for exactly 1 min and immediately rinsed with nanopure water three times for 20 seconds each. Gels were submerged in silver nitrate solution (2 g/L silver nitrate, 0.75 mL/L 37% (v/v) formaldehyde stock solution) for 20 min and immediately rinsed with nanopure water 2 times for 20 sec each. Developing solution (60 g/L sodium carbonate, 0.5 mL/L 37% (v/v) formaldehyde stock solution, 4 mg/L sodium thiosulfate) was used to visualize protein bands by submerging the gels for up to 10 min. Gels were washed with nanopure water twice for 2 min each to terminate the developing process. Finally, gels were submerged in gel fix solution for 10 min and then in 50% (v/v) methanol for 20 min. All staining occurred at room temperature on an orbital shaker.

### *Bioassays*

Diet for the bioassays was prepared by mixing three parts liquid containing treatment or control solution with one part Stonefly *Heliothis* diet (Ward's Science). In addition to the controls outlined for each model system, an additional water control and

Cry1Ab control were prepared fresh at the start of each bioassay. All samples and controls were diluted so that if all protein was fully intact, the final concentration of protein in the diet would be 94.5 ng/g. This value falls between the LC<sub>90</sub> and LC<sub>95</sub> values for the protein and insect population used in the study (see Chapter 2). The use of a high initial concentration will allow for easier observation of any changes in mortality and sub-lethal effects. Diet was mixed thoroughly and 0.3 mL of diet was placed into each well. There were 3 replicates per treatment and 16 insects per replicate (N=48). A single European corn borer (ECB) neonate, *Ostrinia nubilalis*, was placed in each well and the trays were incubated at 27°C ( $\pm$  2°C) and 60-70% humidity. The Corn Insects and Crop Genetics Research Unit of the USDA–ARS (Ames, IA) kindly provided all European corn borers. Larvae were assessed for survival and weight gain after seven days. Bioassays with less than 80% survival in the water controls were reassessed. Treatment replicates were combined and an analysis of variance in SAS 9.3 software (SAS Institute, Cary, NC) was used to determine statistical significance ( $p < 0.05$ ).

#### *ELISAs*

ELISA kits specific for Cry1Ab/1Ac (cat. No. PSP 06200) were purchased from Agdia Inc. (Elkhart, IN). Analysis of samples was performed according to the manufacturer's instructions. Quantitative results were obtained through the use of a standard curve. A Cry1Ab reference standard, provided by Monsanto Company (St. Louis, MO), was used to prepare a fresh 1-20 ng/mL standard curve in the assay buffer, phosphate buffered saline with Tween 20 (PBST), daily. Experiments to validate the specificity and precision of the assay for each model system were carried out to ensure the performance of the assay. Validation of the sensitivity and accuracy of the assay were

not performed; model system samples were analyzed directly, eliminating the need for fortification and recovery or extraction efficiency studies, and all model system samples were diluted to fall in the middle of the quantitative range, thus eliminating the possibility of samples falling below the limit of detection and the lower limit of quantitation.

Specificity, cross-reactivity or interference of the model system components (buffers, enzymes, inhibitors) with the antibodies in the ELISA kit, was determined through buffer matrix testing. Multiple dilutions (1:5,000, 1:10,000, etc.) of only the model system components were prepared using the assay buffer. A 2x standard curve (2-40 ng/mL) was prepared and mixed with equal volumes of the model system dilutions, resulting in a 1x standard curve in the model system buffer. The 1x standard curves in model system buffer were then analyzed with ELISAs in conjunction with a 1x standard curve in assay buffer. The relative percent difference (R%D) was calculated from each point on the standard curve with the following equation:

$$R\%D = \frac{(\text{model system buffer result} - \text{assay buffer result})}{\text{assay buffer result}} \times 100\%.$$

The acceptance criterion

for buffer matrix testing is  $R\%D \leq |15\%|$  between the assay buffer and model system buffer (Schmidt and Alarcon 2011) If the  $R\%D > |15\%|$  across two consecutive points in the standard curve, then there may be matrix effects due to the model system components.

Precision of the ELISA was validated through the use of positive and negative controls, standard curve precision, and dilution agreement. Positive controls (included with the kit) and negative PBST controls were included on every plate. Plates that had standard curve  $R^2 < 0.90$  were re-analyzed. Dilution agreement was used to ensure diluting the model system samples did not affect quantitation of the protein. Standardized

protein was diluted in the model system components (buffers, enzymes, inhibitors) and dilutions were selected to fall above, below, and within the quantitative range. Dilutions were analyzed with ELISAs and percent coefficient of variation (%CV) was calculated across all in-range dilutions with the following equation:

$\%CV = \frac{\text{standard deviation}}{\text{mean adjusted result}} \times 100\%$ . The acceptance criterion for dilution agreement is a  $\%CV \leq 20\%$ . If the  $\%CV > 20\%$ , minimum or maximum dilutions were applied until the  $\%CV$  was  $\leq 20\%$ .

#### *Chymotrypsin*

Trypsin-activated Cry1Ab protein (salt-free, 96% pure) dissolved in 50 mM N-cyclohexyl-3-aminopropanesulfonic acid (CAPS), pH 10.5 was purchased from Case Western Reserve University (Cleveland, OH); the concentration of the protein in solution was 900  $\mu\text{g/mL}$ . Alpha-chymotrypsin from bovine pancreas (cat. No. C4129) was purchased from Sigma-Aldrich (St. Louis, MO). The chymotrypsin model system consisted of a Cry1Ab control, a chymotrypsin control, and three sample replicates (Table 2). A 5:1 mol:mol ratio of enzyme to toxin was prepared by dissolving chymotrypsin in 100 mM potassium phosphate ( $\text{KH}_2\text{PO}_4$ ) buffer, pH 7. All samples were incubated at 37°C for 1 hour or 24 hours. At termination, 150  $\mu\text{L}$  of nanopure water was added to each sample. Three 50  $\mu\text{L}$  aliquots were removed for use in gel electrophoresis, ELISA, and bioassays. All vials were flash frozen in liquid nitrogen and stored at -80°C until analysis.

#### *Long-term degradation in buffer*

The long-term degradation in buffer model system consisted of three sample replicates. One hundred microliters of Cry1Ab solution and 100  $\mu\text{L}$  of 50 mM CAPS was

placed in 1.5 mL vials and stored in the dark at room temperature for 16 weeks. To maintain consistency with the other model systems, 200  $\mu$ L of nanopure water was added to each tube at termination. Three 50  $\mu$ L aliquots were removed for use in gel electrophoresis, ELISA, and bioassays. All vials were flash frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until analysis.

### *Photodegradation*

The photodegradation model system consisted of two treatments and three sample replicates per treatment. Each sample 115  $\mu$ L of Cry1Ab solution placed in a quartz NMR tube. The samples were then placed in a Rayonet photoreactor (Southern New England Ultraviolet Company, Branford, CT) and irradiated with 254 nm light for either 2.5 or 5 min. Following irradiation, 75  $\mu$ L of sample was transferred into a separate vial and 225  $\mu$ L of water was added. Three 50  $\mu$ L aliquots were removed for use in gel electrophoresis, ELISA, and bioassays and all vials were flash frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until analysis.

### *Acidic buffer*

The acidic buffer treatment (pH 1.64) consisted of four time points, with three samples at each time point. All samples consisted of 75  $\mu$ L Cry1Ab solution (in CAPS, pH 10.5) and 75  $\mu$ L  $\text{KH}_2\text{PO}_4$  (pH 1.3). At each time point, one sample was held at room temperature, while the remaining two samples were held at  $40^{\circ}\text{C}$ . All samples were terminated with 150  $\mu$ L nanopure water at 4, 5, 6, or 7 days. After termination, samples were flash frozen with liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until analysis with gel electrophoresis.

### *Proteinase K*

Proteinase K from *Tritirachium album* was purchased from Sigma-Aldrich and a 1:1 mol:mol ratio of enzyme to toxin was prepared by dissolving proteinase K in 20 mM Tris (hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, pH 7.5. The proteinase K model system consisted of a Cry1Ab control, a proteinase K control and three sample replicates (Table 3). All samples were incubated at 37°C for 30 minutes. At termination, 150 µL of nanopure water was added to each sample and then three 50 µL aliquots were removed for use in gel electrophoresis, ELISAs, and bioassays. All vials were flash frozen in liquid nitrogen and stored at -80°C until analysis.

Following analysis of the proteinase K treatments, a mass digest of Cry1Ab was set up to generate sufficient quantities of fragments for additional analysis. For this mass digest, 4.5 mL of Cry1Ab was incubated with 4.5 mL of proteinase K at 37°C for 30 minutes. At termination, 9 mL of nanopure water was added aliquots of 50 µL, 300 µL, and 1 mL were prepared, and all aliquots were flash frozen in liquid nitrogen and stored at -80°C. A Cry1Ab control (4.5 mL Cry1Ab and 4.5 mL 20 mM Tris-HCl; terminated with 9 mL water) and a proteinase K control (4.5 mL 50 mM CAPS and 4.5 mL proteinase K solution; terminated with 9 mL water) were also prepared and subjected to the same conditions. Gel electrophoresis was performed on an aliquot of the mass digest to confirm the degradation pattern remained the same.

An aliquot of the mass digest was subjected to gel electrophoresis with a tris-tricine gel; the use of a tris-tricine gel allowed for resolution of fragments with low molecular weights. Tris-tricine gels (16.5% acrylamide, cat. no. 456-3064), running buffer (cat. no. 161-0744), sample buffer (cat. no. 161-0739) and polypeptide SDS-PAGE

standards (cat. no. 161-0326) were purchased from Bio-Rad Laboratories Inc. and prepared according to the manufacturer's directions. Equal volumes of sample and sample buffer were combined and heated at 95°C for 5min, chilled in ice for 10 min, and centrifuged at 12,000 x g for 5 min. Twenty microliters of sample was loaded into the lanes and a constant 100 volts was applied to the gel until the dye front was within 1 cm of the bottom of the gel (approximate run time was 2 hours). The gel was stained with silver stain and digitized as previously described.

A separatory column was used to isolate fragments for further analysis. Sephadex® G-50 separatory media was purchased from Sigma-Aldrich and placed in excess 50 mM sodium phosphate buffer (with 15 mM NaCl, pH 7.0) overnight to allow the beads to swell. Suspended particles were decanted and the media was degassed for 15 minutes with occasional swirling. Glass wool was placed in the bottom of a 1 cm diameter column and 0.5 cm of sand was added to prevent column blockage. The column was filled 1/3 full with fresh buffer and the gel was poured in one continuous step until the desired height of 12.7 cm was reached (10 mL approximate column volume). The gel was allowed to settle for 10 min and then the outlet was opened to achieve even sedimentation; fresh buffer was continuously added to ensure the column did not run dry. Following sedimentation, the outlet was closed and excess buffer was removed with a Pasteur pipet. A 3 mL volume of sample was added via Pasteur pipet and the outlet was opened to allow the sample to enter the column bed, at which time the outlet was closed. Additional sample buffer was carefully added with a Pasteur pipet, the outlet was opened, and fraction collection was initiated. Fifteen 1 mL fractions were collected from the three



mass digest samples. A Nano-Drop® ND-1000 spectrophotometer was used to determine protein concentration in each fragment by recording absorbance at 280 nm.

Following separation of the fragments, ELISAs were performed on selected fractions for all three samples. The total amount of protein in the fractions varied from fraction to fraction. To account for this variation, the protein concentration determined by Nano-Drop® spectrophotometry (Table 4) was used as a starting point, and all fractions were appropriately diluted so that the final concentration of total protein in each fraction was 11.25 ng/mL for the ELISA.

## Results

### *Chymotrypsin*

The chymotrypsin model system produced minor degradation of Cry1Ab at 1 hour (Figure 1A). Significantly more degradation of the protein was observed at 24 hours (Figure 1B); chymotrypsin was almost completely degraded at 24 hours, so longer incubations were not investigated. There was no difference in the number of fragments produced by the different incubations times; both yield two fragments, approximately 25 and 34 kDa in size.

Buffer matrix testing indicated there would be no effects from the buffer at a 1:22,500 dilution of the samples (Table 5). Dilution agreement testing determined that diluting the samples in the range of 1:15,000 to 1:50,000 would not affect protein quantitation (%CV = 8.00) (Table 6). Therefore, all samples from the chymotrypsin model system were diluted by a factor of 1:22,500 prior to ELISA analysis. At 1 hour, ELISA results indicated that very little degradation of the protein occurred; 95-110% of the parent protein remained (Table 7). After 24 hours, ELISA results indicated the

Cry1Ab protein had been significantly degraded with only 21-68% of the parent protein remaining. Average survival for insects in the 1 hour treatments was 34.7% and 77.6% for the 24 hour treatments (Figure 2). The average weight of the insects was higher in the 24 hour treatment (0.90 mg) compared to the 1 hour treatment (0.34 mg).

#### *Long-term degradation in buffer*

The long-term degradation of Cry1Ab in only CAPS buffer produced multiple protein fragments (Figure 3). The majority of the fragments produced were three fragments approximately 27, 40, and 45 kDa; several fragments 25 kDa or smaller were also visualized. Buffer matrix testing determined that there would be no significant effects from the buffer in the dilution range of 1:10,000 – 1:50,000 (Table 8). Dilution agreement testing indicated that protein quantitation would not be affected if the samples were diluted in the range of 1:15,000 – 1:50,000 (%CV = 4.85)(Table 9). A dilution of factor of 1:22,500 was subsequently used to dilute all samples by prior to ELISA analysis. Since both the long-term degradation model system and the photodegradation model system consisted of only CAPS and water, the results from the buffer matrix and dilution agreement tests were also used to determine the dilutions used in the photodegradation model system.

ELISA results indicated that significant degradation of the protein occurred; approximately 50% of the fully intact protein remained (Table 10). The average larval survival for the treatments (70.1%) was slightly lower than the water control survival; however, average larval weight was greatly reduced compared to the water control, 0.75 mg and 6.60 mg respectively (Figure 4).

### *Photodegradation*

Significant degradation of Cry1Ab occurred at both time points, but fully intact protein remained (Figure 5). A greater number of fragments and higher quantities of all fragments were present in the 5 min samples than the 2.5 min samples. Significant smearing was observed in the gel in all samples. The smearing was contained above the 100 kDa, and thus, it did not interfere with fragment visualization. All samples were diluted by a factor of 1:22,500 prior to ELISA analysis, as determined previously (see long-term degradation in buffer results). Cry1Ab protein was detected at a low level by ELISA in only one of three 2.5 min samples and was not detected in any of the 5 min samples (Table 11), despite the presence of small amounts of Cry1Ab in all samples, as indicated by the gel (Figure 5). There were no significant differences between the 2.5 and 5 min samples and the water control for larval survival; survival in all three was greater than 97% (Figure 6). Fragments of the photodegradation model system do not appear to have sub-lethal effects as no significant differences are present between the water control and the 2.5 min samples. Average weight in the 5 min treatments was significantly higher than the water control, suggesting a possible increase in growth, but this was not investigated further.

### *Acidic buffer*

Minimal degradation was observed in the four room temperature controls. Significant degradation of the protein was observed in the 40°C treatments at all four time points, with nearly all the parent protein completely degraded after 7 days (Figure 7). There were a larger number of different protein fragments in the treatments, but the quantities of each fragment were low; the prominent band for most samples is a band

near 5 kDa. Buffer matrix testing indicated that the buffer would not have significant effects on the ELISA results, if the samples were diluted in the 1:10,000 - 1:1,000,000 range (Table 12). Protein quantitation would be unaffected if all samples were diluted in the 1:10,000 - 1:100,000 range; for consistency, a dilution factor of 1:22,500 was applied to all samples prior to analysis with ELISA (Table 13). No fully-intact Cry1Ab was detected in any of the room temperature controls or the 40°C treatments by ELISA (Table 14). Bioassays of the 4 and 5 day samples and the 6 and 7 day samples were performed on different days. Larval survival in all model system controls and treatments was high, >80%, and there were no significant differences when compared to the water control (Figure 8). Average weight of the insects in the 4 day room temperature control was low, suggesting possible sub-lethal effects, but this difference was not statistically significant. Average weight of the insects in the 6 and 7 day 40°C treatment was significantly lower than the water control, suggesting possible sub-lethal effects. All other treatments and controls were either not significantly different from the water control, or were statistically higher than the water control.

#### *Proteinase K*

The proteinase K model system completely degraded the Cry1Ab protein (Figure 9A). The resulting degradation pattern was dominated by a fragment or multiple fragments in the 5 kDa range. Smaller quantities of additional fragments in the 10-15 kDa range were also present. Buffer matrix testing indicated that buffer components would not significantly affect ELISA results (Table 15). Dilution agreement testing determined that diluting the samples in the 1:15,000 to 1:50,000 range would not affect protein quantitation (%CV = 11.11) (Table 16). Based on the buffer matrix and dilution

agreement results, all proteinase K model system samples were diluted by a factor of 1:20,000 prior to ELISA analysis. ELISA results of the proteinase K treatments indicated that the Cry1Ab fragments were still highly detectable by the ELISA antibodies (Table 17A).

Significant biological effects were observed in all treatments. These bioassays were performed a second time and the combined data are presented (Figure 10A) (N=96). Survival in the treatments (52.4%) was comparable to the Cry1Ab controls (47-60%) and was significantly lower than the water and proteinase K controls (95% and 92% respectively). Average weight of the insects fed on diet containing the treatment solutions (0.33 mg) did not differ from the Cry1Ab controls (0.32 – 0.54 mg), but was significantly lower than the water and proteinase K controls (6.61 and 6.35 mg, respectively).

Since the results of the gel electrophoresis (protein is highly degraded) and ELISAs and bioassays (protein still detectable and biological active) contradicted each other, the entire proteinase K model system was performed a second time. The results from the gel electrophoresis (Figure 9B), ELISAs (Table 17B), and bioassays (Figure 10B) supported the previous findings.

Proteinase K treatments from the first run were silver stained (Figure 9A) in an attempt to visualize fragments that might not have been captured by Coomassie staining. The silver staining procedure did detect additional bands in the treatments; however, all of these bands appeared to correspond to additional bands visualized in the proteinase K control (Figure 9C).

Gel electrophoresis on the mass digest (Figure 11A) indicated a similar degradation pattern as previously observed (Figure 9). Gel electrophoresis with the tris-

tricine gel adequately resolved the low molecular weight fragments (Figure 11B).

Although many of the fragments in the treatment corresponded to bands in the proteinase K enzyme control, two bands in the treatment between 3.5 kDa and 6.5 kDa did not appear elsewhere; thus, the proteinase K digestion appears to produce at least two fragments that have low molecular weights.

A G-50 separatory column was used to fractionate the treatment protein and the proteinase K control and Cry1Ab protein control. Fraction analysis by Nano-Drop® spectrophotometry yielded six fractions of interest in the treatment. High concentrations of protein were observed in fractions 4-7 and 12-13 in the treatment (Figure 12). The Cry1Ab control peaked across fractions 5-6, while no discernable peaks were observed in the proteinase K control. ELISAs were performed individually on fractions 4-7 and 12-13 for the treatment and the two control samples. The ELISA results indicated that Cry1Ab was present in treatment fractions 4-7 and in Cry1Ab control fractions 5-7; no protein was detected in any of the proteinase K control fractions (Table 4).

## **Discussion**

The gel electrophoresis, ELISA, and bioassay results in four of the five model systems tested corroborated each other. For the chymotrypsin model system, at 1 hour, very few fragments are observed in the gel and most of the parent protein remains intact (Figure 1A). ELISA results also indicate that similar amounts of parent protein are present in the treatments and the control. Bioassays suggest that more ECB larval survival occurs in the 1 hour treatments than the Cry1Ab control, though the cause of this is unknown. At 24 hours, the gel electrophoresis and ELISA results indicate that significant losses of the parent protein occurred. The bioassay results show significant

increases in larval survival; however, average larval weights are still suppressed. Thus, at 24 hours, sufficient amounts of fully intact Cry1Ab remain to affect larval growth, but the quantities present are below the threshold needed for mortality to occur. Since the biological activity and ELISA detectability of the protein decreases as the protein becomes fragmented, the conclusion can be drawn that Cry1Ab fragments generated by the chymotrypsin model system are not detectable by ELISA and do not retain any bioactivity.

For the long-term degradation in buffer model system, multiple fragments of Cry1Ab were generated, but substantial quantities of the fully intact protein remained (Figure 3). The ELISA results indicate approximately half of the protein is no longer present (Table 10). Increased larval survival in the treatments suggests that substantial degradation of the protein occurred, but the low average weight of the insects in the treatments indicates that enough protein remains intact to cause sub-lethal effects (Figure 4). Based on these results, the conclusion can be drawn that the fragments generated by the long-term degradation in buffer model system are not detectable by ELISAs and do not retain bioactivity.

In the photodegradation model system, numerous fragments were produced following 2.5 and 5 min exposure to UV light (Figure 5). Molecules larger than the 67 kDa parent protein were also visible in all lanes. These larger molecules are most likely the result of two Cry1Ab molecules (or fragments) becoming cross-linked to each other and forming dimers or other complex molecules. Protein-protein cross-linking is known to occur at 254 nm (Martinson *et al.* 1976). Cry1Ab was detected in only one of six treatments by the ELISA, despite the presence of fully-intact protein still present in all

lanes (Table 11). Detrimental effects of the fragments were not observed in the bioassays (though the 5 min treatments seemed to promote insect growth)(Figure 6). This suggests that the structure of these fully-intact proteins may have been altered by the UV light to prevent antibody binding. Disulfide bonds can absorb light between 250-300 nm, which can lead to photo-dissociation of cystine into two thiyl radicals. These radicals can dimerize (explaining the larger molecules observed in the gel) or they may react with oxygen, resulting in an irreversible oxidation of the disulfide bond. This oxidation reaction can compromise the tertiary structure of the protein, and result in decreased antibody binding (from ELISAs) and decreased toxicity (Davies and Truscott 2001). From these results, the conclusion can be drawn that the fragments generated by the photodegradation model system do not retain bioactivity and are not detectable by ELISA.

The acidic buffer model system produced numerous fragments over the 4 to 7 day degradation, including nearly complete degradation of the protein at 7 days (Figure 7). However, the quantity of fragments produced never increased, and the amounts of individual fragments did not accumulate, suggesting continued degradation of the fragments. Fragments of the acidic buffer model system are not detectable by ELISA (Table 14). These fragments do not appear to retain bioactivity, as survival in all treatments did not differ from the water control (Figure 8). Survival in the room temperature controls also was not significantly different from the water control, though significant amounts of parent protein remained as observed in the gel (Figure 7). The acidic conditions in these controls and treatments possibly caused denaturing of the protein, resulting in a loss of bioactivity and detectability by ELISA. Denaturing of



proteins is known to occur at low pH (Goto *et al.* 1990). In some cases, denatured proteins may refold into a molten globule-like conformation (Goto *et al.* 1990, Fink *et al.* 1994), but the exact conformational state of the proteins and fragments in the controls and treatments was not investigated in the study and remains unknown. Decreased weight of the insects in the 6 and 7 day treatments indicate sublethal effect may be occurring (Figure 8B). The average weight in these treatments was significantly higher than the average weight in the Cry1Ab control; the average weight in other model systems where survival was not impacted but sub-lethal effects existed (such as the proteinase K model system) were not statistically different from the Cry1Ab control (Figure 10). This suggests that the effects observed in the 6 and 7 day treatments might not be attributable to Cry1Ab fragments, but rather to buffer components. The average weight in the 5 day treatment was statistically higher than the water control, further supporting the possibility that variation in the biological systems is responsible for the weight differences.

Although additional bioassay replicates would provide more information on this issue, no further studies were performed to address this issue, due to a lack of promising results in the ELISAs and survival in the bioassays. Based on the results of the acidic buffer model system, the conclusion can be drawn that the fragments generated by this model system are not detectable by ELISA and do not appear to retain any biological activity.

For these four model systems, the conclusion can be drawn that the fragments generated by the model systems are not detectable by ELISA, since the concentrations of Cry1Ab in the treatment solutions decreased. If the fragments were detectable by the ELISA, it would be expected that the concentrations of Cry1Ab, as determined by the

ELISA, would remain unchanged or even increase, if multiple fragments capable of binding with the antibodies were formed from the degradation of a single protein.

The proteinase K model system produced different results, because initial tests on the fragments generated by the proteinase K model system suggests the fragments are still detectable by ELISA and retain bioactivity (Figure 10, Table 17). The model system digestion was repeated, and the results of the first digestion were replicated (Figures 9B, 10B, Table 17B). These positive ELISA and bioassay results were unexpected given the low molecular weight of these fragments (Figure 11B). A separatory column was used in an attempt to isolate separate fragments and test each fragment or a smaller collection of fragments with ELISAs. This separation resulted in six fractions of interest: fractions 4-7 and 12-13 (Figure 12). The largest peak in the treatment was centered across fractions 4-7, which aligned with the peak in the Cry1Ab control. This is surprising because the Sephadex® G-50 media used in the separatory column has a cutoff of approximately 30 kDa; proteins larger than 30 kDa should elute in the first few fractions, while proteins smaller than 30 kDa should elute in later fractions. This is true for the Cry1Ab control, as the fully intact protein (67 kDa) elutes early in the column (fractions 5-6, Figure 12). This suggests that high molecular weight proteins (>30 kDa) are still present in the treatment; however, no proteins >30 kDa are observed in the gels (Figures 9, 11B).

One possible explanation for why the fragments are still detectable by ELISA is that the proteinase K fragments may still be immunoreactive with the ELISA antibodies. Lutz *et al.* (2005) detected Cry1Ab with ELISA in gastrointestinal tract samples from cattle fed transgenic maize; however Western blotting indicated that only 17 and 34 kDa fragments were present. Emmerling *et al.* (2011) detected small amounts of Cry1Ab with

ELISA in hindgut and cast material samples from earthworms; Western blotting did not detect any fragments, leading the authors to speculate that the fragments detected by the ELISA were smaller than 17 kDa. The fragments in the current study meet these criteria as they are believed to be between 3.5 and 6.5 kDa in size. However, this does not explain why the fragments move through a separatory column in a manner similar to larger proteins or why the fragments still exhibit biological activity. Some fragments of Cry proteins are known to retain biological activity. Diaz-Mendoza *et al.* (2007) reported that 43 and 46 kDa fragments of Cry1Ab (69 kDa active form) generated by a trypsin purified from the Mediterranean corn borer, *Sesamia nonagrioides*, were still toxic to *S. nonagrioides* larvae. However, no other known studies have tested the bioactivity of Cry protein fragments with very low molecular weights; it seems unlikely that a highly degraded fragment (17 kDa or less) would retain enough structure to bind to and insert into an insect's midgut cells, and ultimately, result in toxicity.

Another possible explanation is that proteinase K degrades the protein, but the fragments then re-associate with each other, possibly through disulfide bridges. Proteinase K cleaves at the carboxylic group of aliphatic and aromatic amino acids with blocked  $\alpha$ -amino groups; disulfide bridges may not be affected by this cleavage (Ebeling *et al.* 1974). Fragments may be visualized in gel electrophoresis because the denaturing conditions in SDS-PAGE break the disulfide bonds, causing the fragments to dissociate from each other. If the fragments do re-associate with each other, they would have a higher overall molecular weight, which would cause them to pass through the separatory column quickly and elute in earlier fractions, similar to fully-intact, large molecular weight proteins. This also may explain the positive ELISA and bioassay results; the

association of the fragments may allow the protein to retain enough of its native conformation to allow for antibody binding (resulting in positive ELISA detections) and binding to the insect midgut (resulting in toxicity).

Two studies observed similar processes with Cry4A and Cry11A. Two Cry4A fragments of 20 and 45 kDa were formed by *in vitro* and *in vivo* processing (Yamagiwa *et al.* 1999). Individually, the two fragments exhibited no toxicity against *Culex pipiens* larvae; however, when the two fragments were mixed together, significant toxicity was observed. The authors proposed that the two fragments associated with each other to form an insecticidal complex since the fragments could not be separated by gel filtration; fragment association was confirmed with co-precipitation experiments (Yamagiwa *et al.* 1999). Similar results were observed with Cry11A for which a 32- and a 36-kDa fragment had no toxicity individually, but exhibited significant toxicity when expressed together (Yamagiwa *et al.* 2004). The two fragments also eluted together in size-exclusion chromatography, and co-precipitation experiments determined that they were associated with each other (Yamagiwa *et al.* 2004).

A similar inability to resolve the fragments with gel filtration was observed in the current study, supporting the theory that the fragments were associated with each other. More research is needed to confirm this association of fragments. First, fragments should be separated further to allow for identification and sequencing of individual fragments. Once sequenced, fusion proteins containing fragments can be synthesized and used in co-precipitation and toxicity experiments, similar to those described in Yamagiwa *et al.* (1999). In that study, fusion proteins of glutathione *S*-transferase (GST) linked to the fragments or fully-intact protein were used in a co-precipitation experiment to show that

the fragments associated with each other. The GST-fragment fusion proteins were also tested for toxicity individually and together; toxicity was observed when the fragments co-existed, and no toxicity was observed when the fragments were tested individually (Yamagiwa *et al.* 1999).

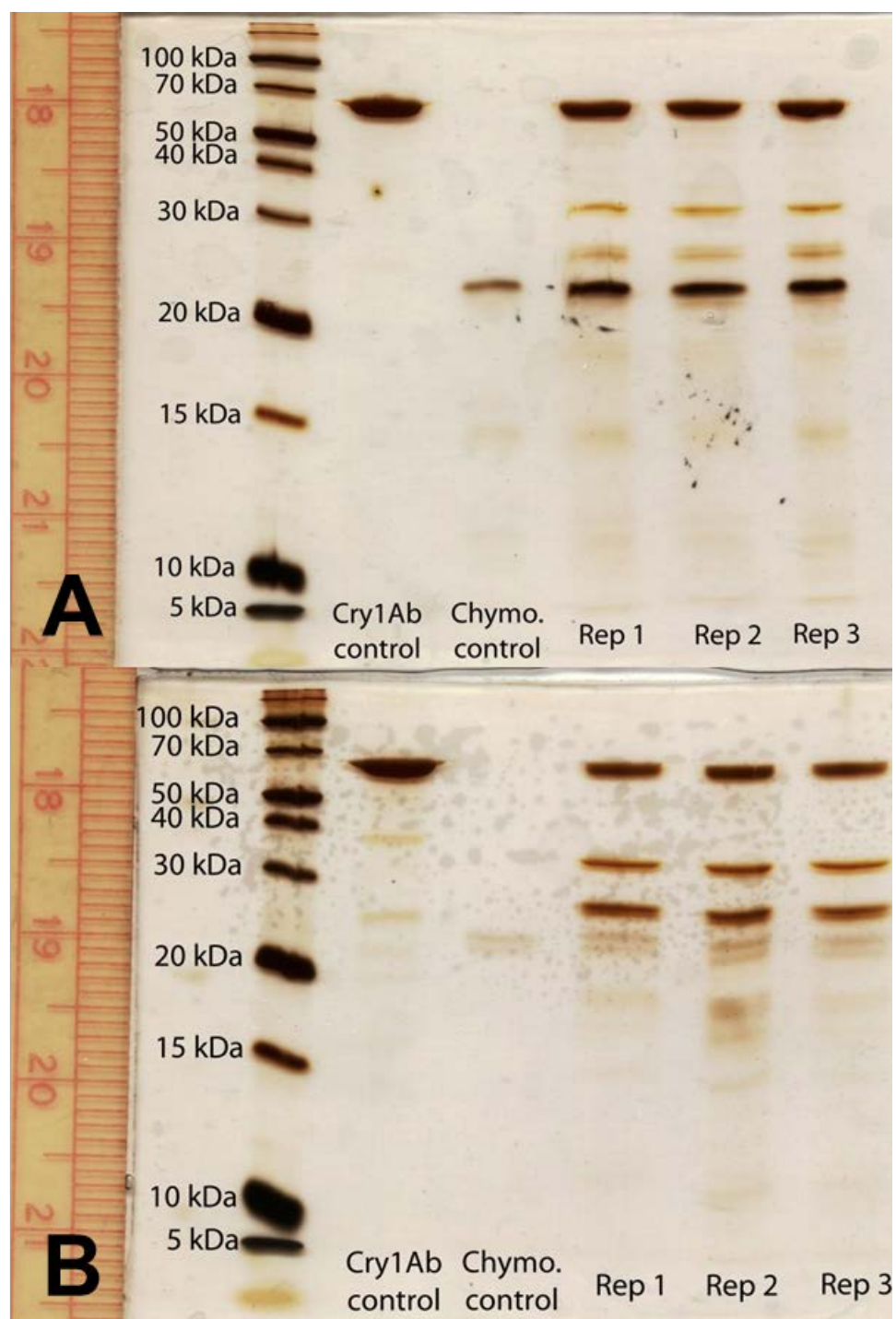
## Conclusions

Five model systems were used to generate fragments of Cry1Ab. In four of these model systems, the chymotrypsin model system, acidic buffer model system, photodegradation model system, and long-term degradation in buffer model system, the fragments generated were not detectable by ELISA and did not retain bioactivity. In the proteinase K model system, the fragments generated were detectable by ELISA and retained bioactivity. The reason for this is unknown; more research is needed to understand this phenomenon. Nevertheless, based on the results described here, researchers do not appear to be over-estimating the amount of Cry proteins in the environment, as fragments that are detectable by ELISA, also retain bioactivity, and those fragments not detectable by ELISA have no biological activity. However, only a few mechanisms of Cry protein degradation were investigated. Additionally, an ELISA from only one manufacturer was used. Other manufacturers may use different host organisms to produce antibodies for their ELISA kits, and these antibodies may bind to different epitopes on the fragmented protein, potentially generating different results. Thus, more research is needed with other ELISA kits and additional model systems, especially microbe-based model systems, to obtain a better understanding of the fate of Cry proteins in the environment.

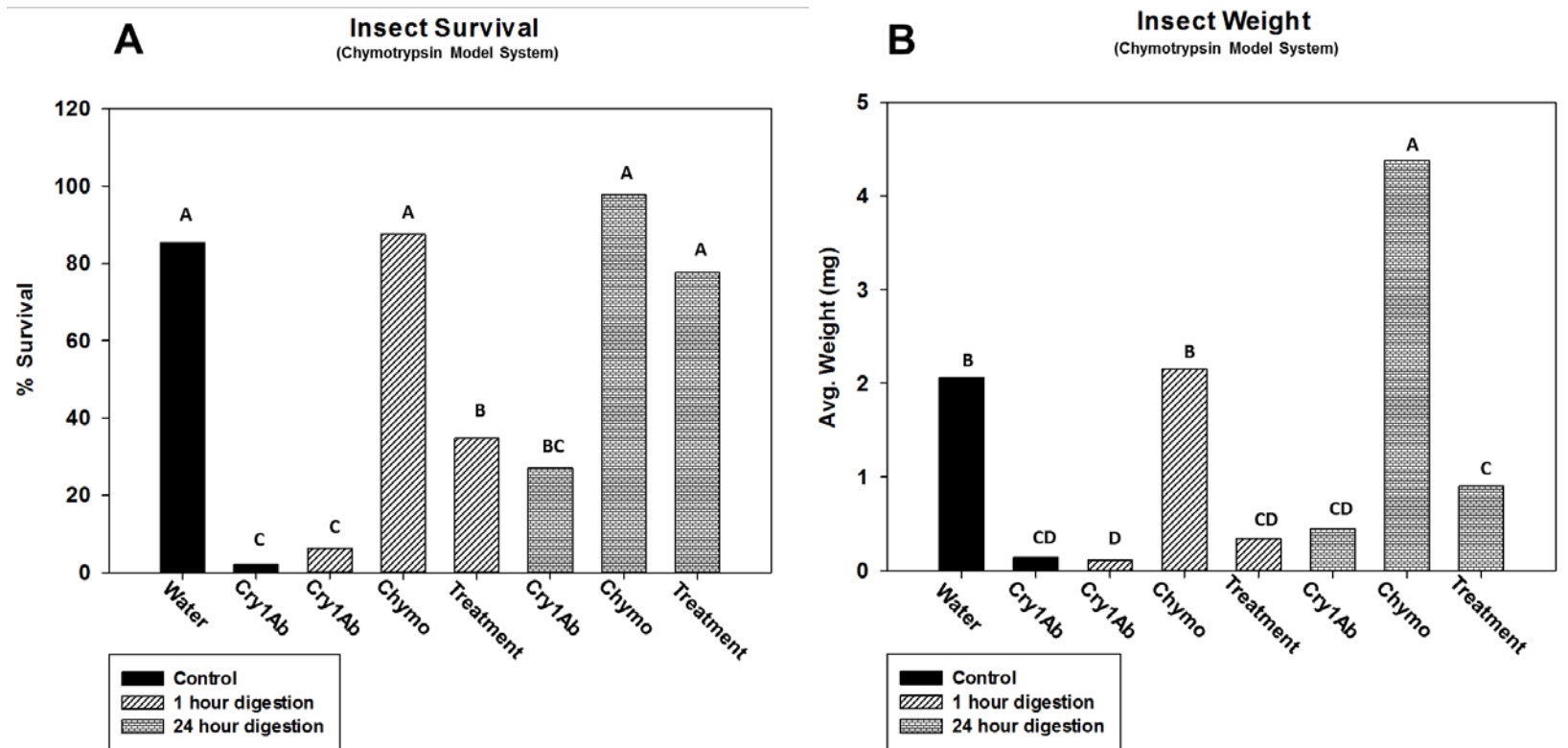
## **Acknowledgements**

We thank Jennifer Anderson, Courtney Davis-Vogel, and Anita Unger at DuPont Pioneer for providing training on the ELISA procedure and method validation and Chad Boeckman and Kris Sturtz at DuPont Pioneer for providing training with the bioassay procedure. We thank William Jenks for access to the Rayonet photoreactor for the UV photodegradation studies. We also thank Keith Bidne with the USDA–ARS Corn Insects and Crop Genetics Research Unit for kindly providing the European corn borer larvae used in the study. Mention of a proprietary product does not constitute an endorsement or a recommendation by Iowa State University or USDA for its use.

## Figures

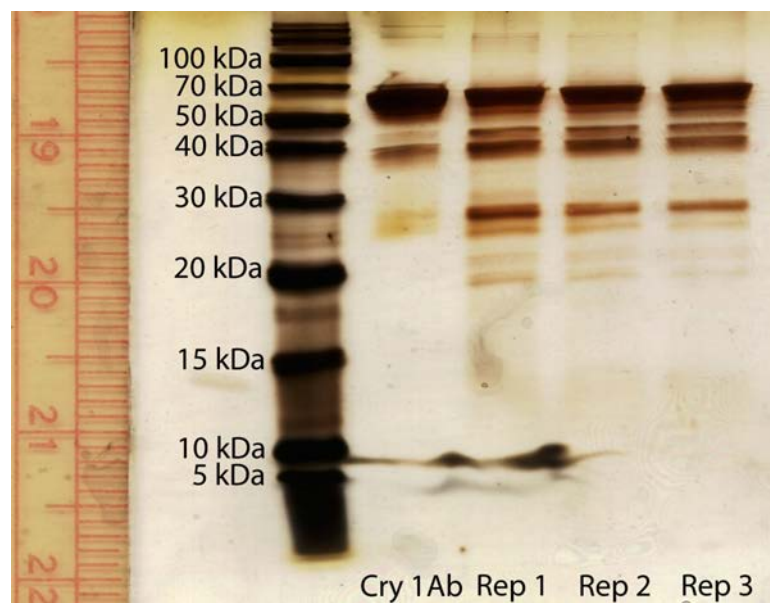


**Figure 1.** Degradation of Cry1Ab with the chymotrypsin model system. Incubation times: **A** – 1 hour, **B** – 24 hours. Cry1Ab control – Cry1Ab only; Chymo. control – chymotrypsin in buffer only; Rep 1-3 – treatments containing Cry1Ab and chymotrypsin.

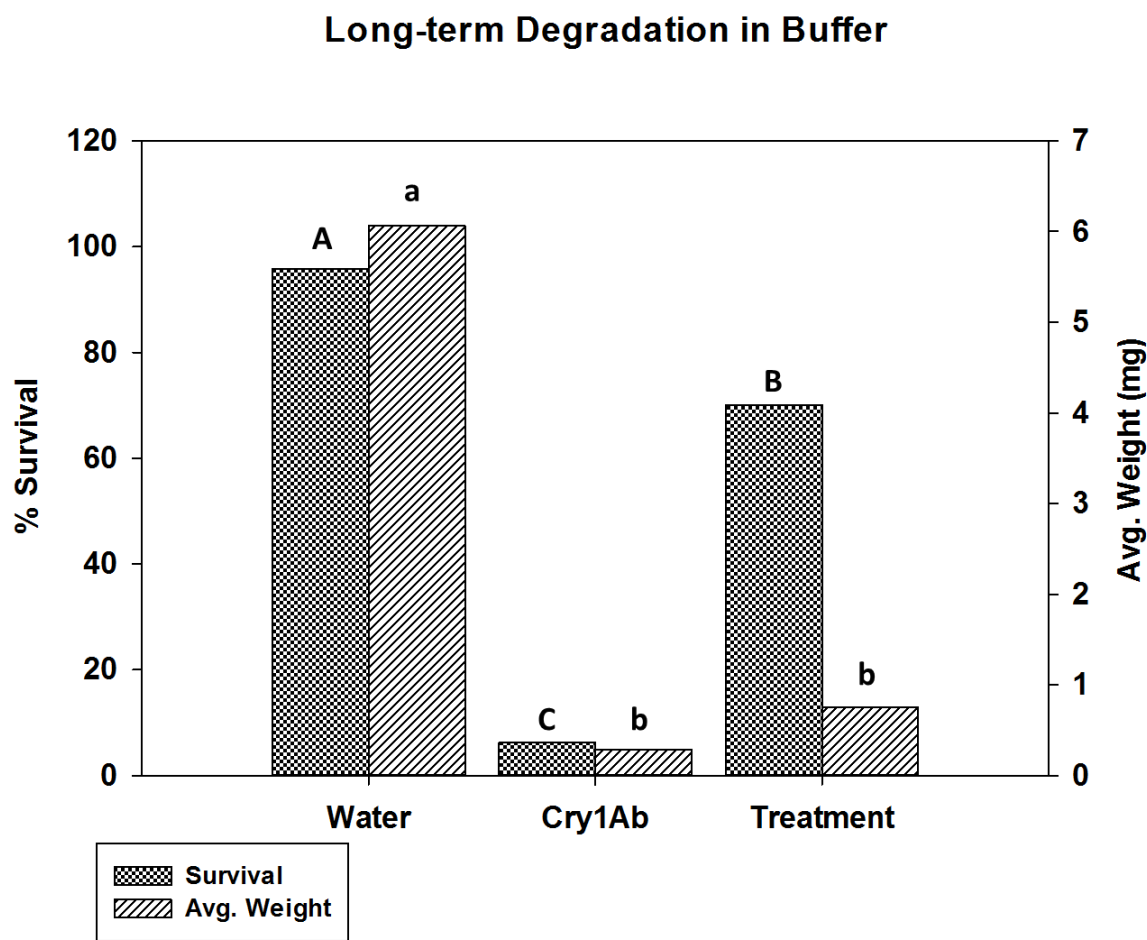


**Figure 2.** **A** - Survival of insects fed on diet prepared from the chymotrypsin model system. **B** - Average weight of insects fed on diet prepared from the chymotrypsin model system. The determination of significance required the data to be log transformed due to a low number of individuals in some of the treatments. Cry1Ab = Cry1Ab only; Chymo = chymotrypsin in buffer only; Treatment = treatments containing Cry1Ab and chymotrypsin. Letters indicate significance across treatments ( $p = 0.05$ ).

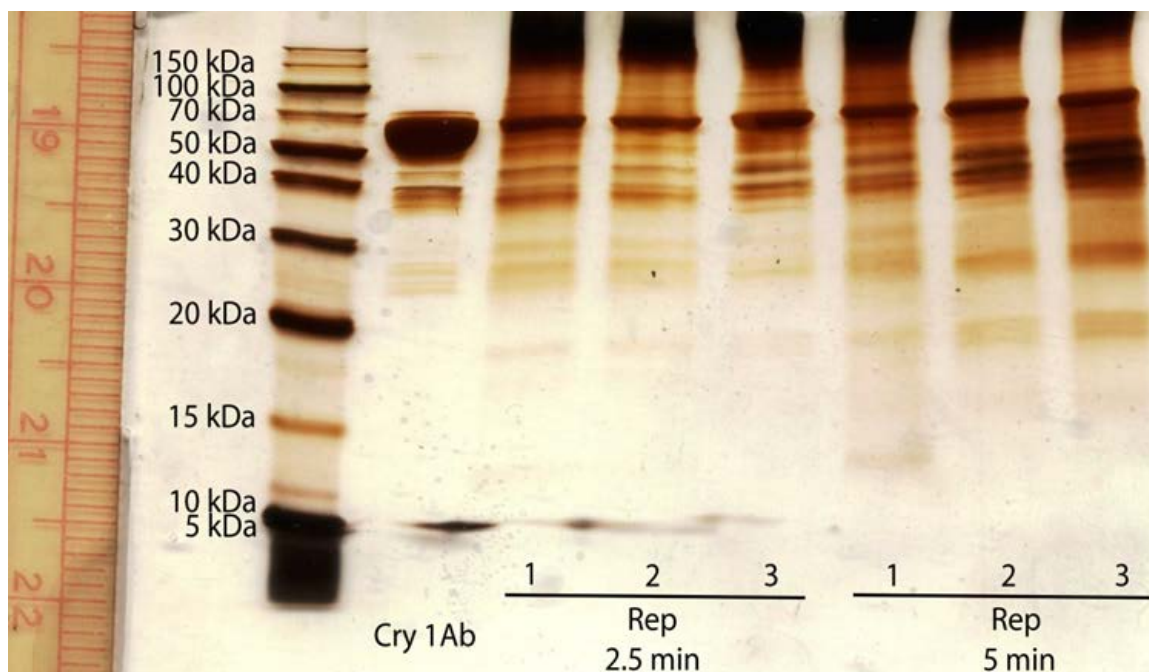




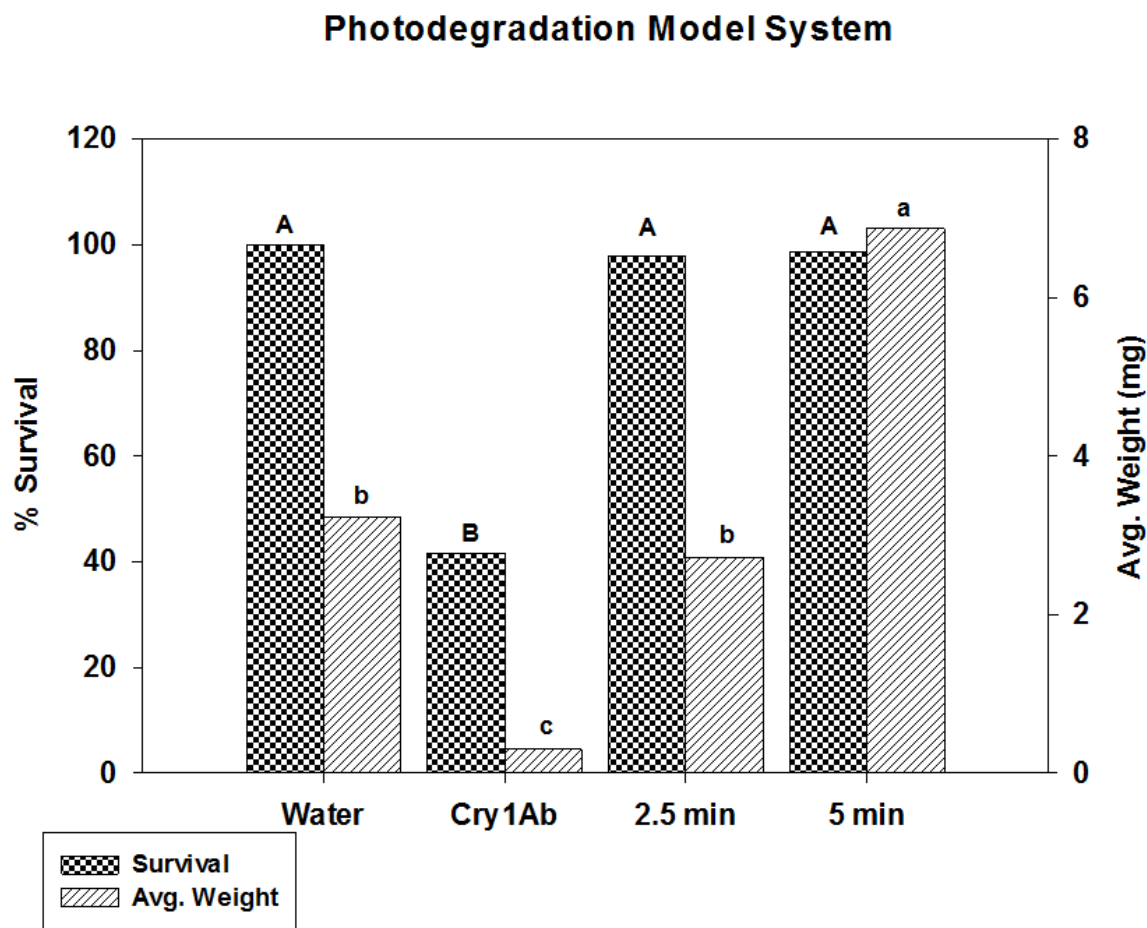
**Figure 3.** Degradation of Cry1Ab with the long-term degradation in buffer model system after a 16-week incubation. Cry1Ab = freshly prepared protein; Rep 1-3 = treatments containing Cry1Ab in 50 mM CAPS buffer.



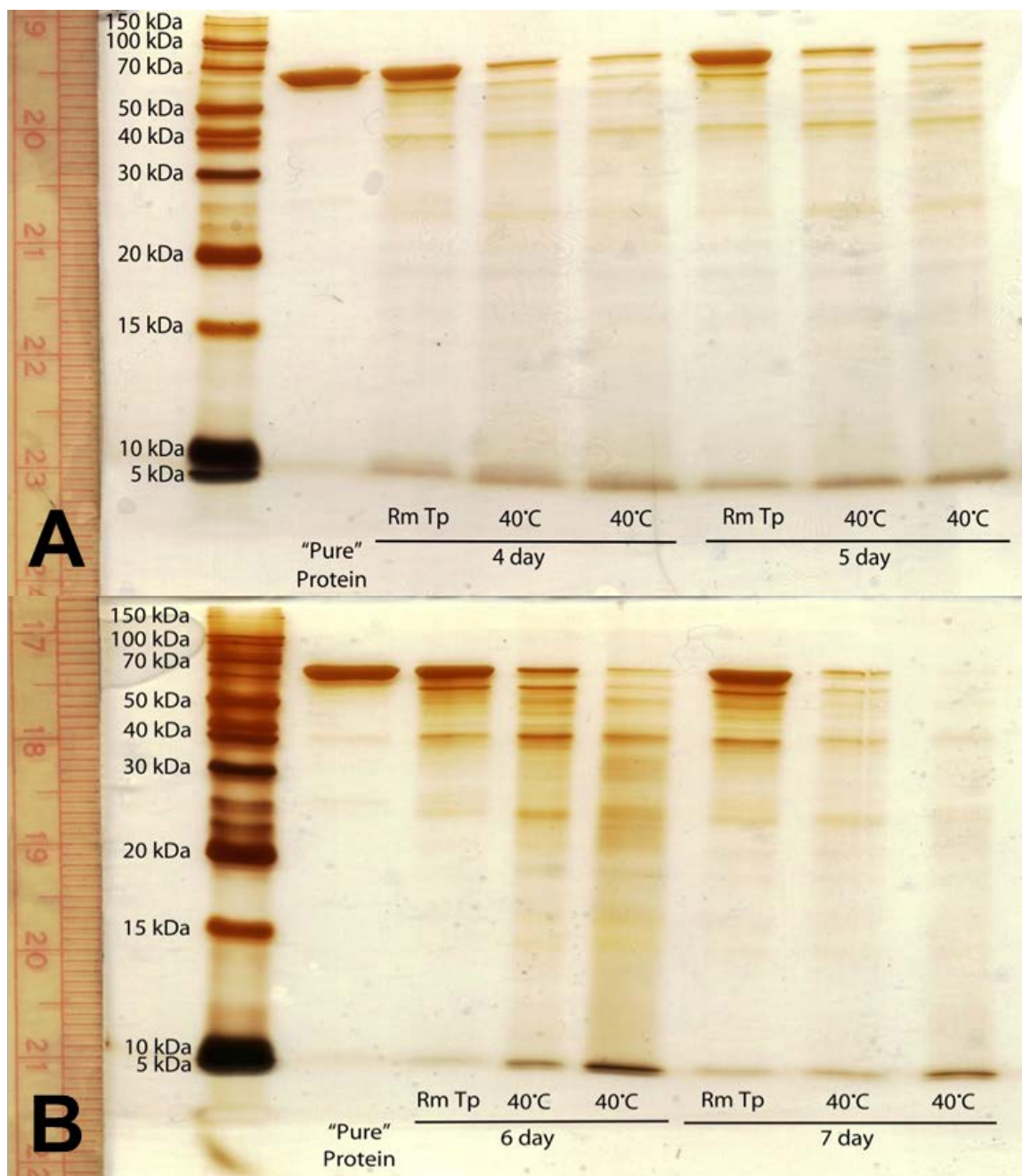
**Figure 4.** Survival and average weight of surviving insects fed on diet prepared from the long-term degradation model system. Cry1Ab = Cry1Ab only; Treatment = treatments containing Cry1Ab degraded over 16 weeks at room temperature in 50 mM CAPS buffer, pH 10.5. Uppercase letters indicate significance within survival across treatments ( $p = 0.05$ ). Lowercase letters indicate significance within average weight across treatments ( $p = 0.05$ ).



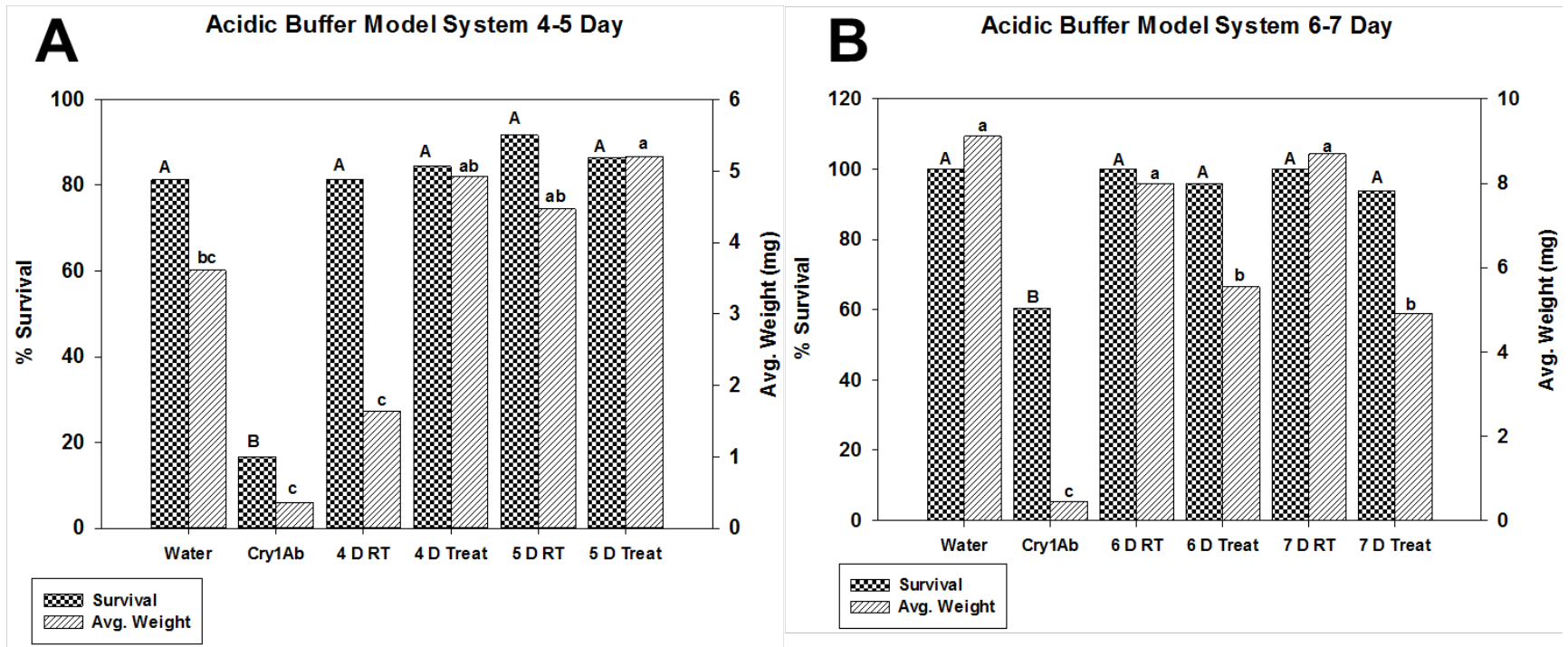
**Figure 5.** Degradation of Cry1Ab with the photodegradation model system. Samples were exposed to 254 nm light for 2.5 or 5 minutes. Cry1Ab = freshly prepared protein; Rep 1-3 = treatments containing Cry1Ab in 50 mM CAPS buffer.



**Figure 6.** Survival and average weight of surviving insects fed on diet prepared from the photodegradation model system. Cry1Ab = Cry1Ab only; 2.5 min = 2.5 min exposure to 254 nm light; 5 min = 5 min exposure to 254 nm light. Uppercase letters indicate significance within survival across treatments ( $p = 0.05$ ). Lowercase letters indicate significance within average weight across treatments ( $p = 0.05$ ).

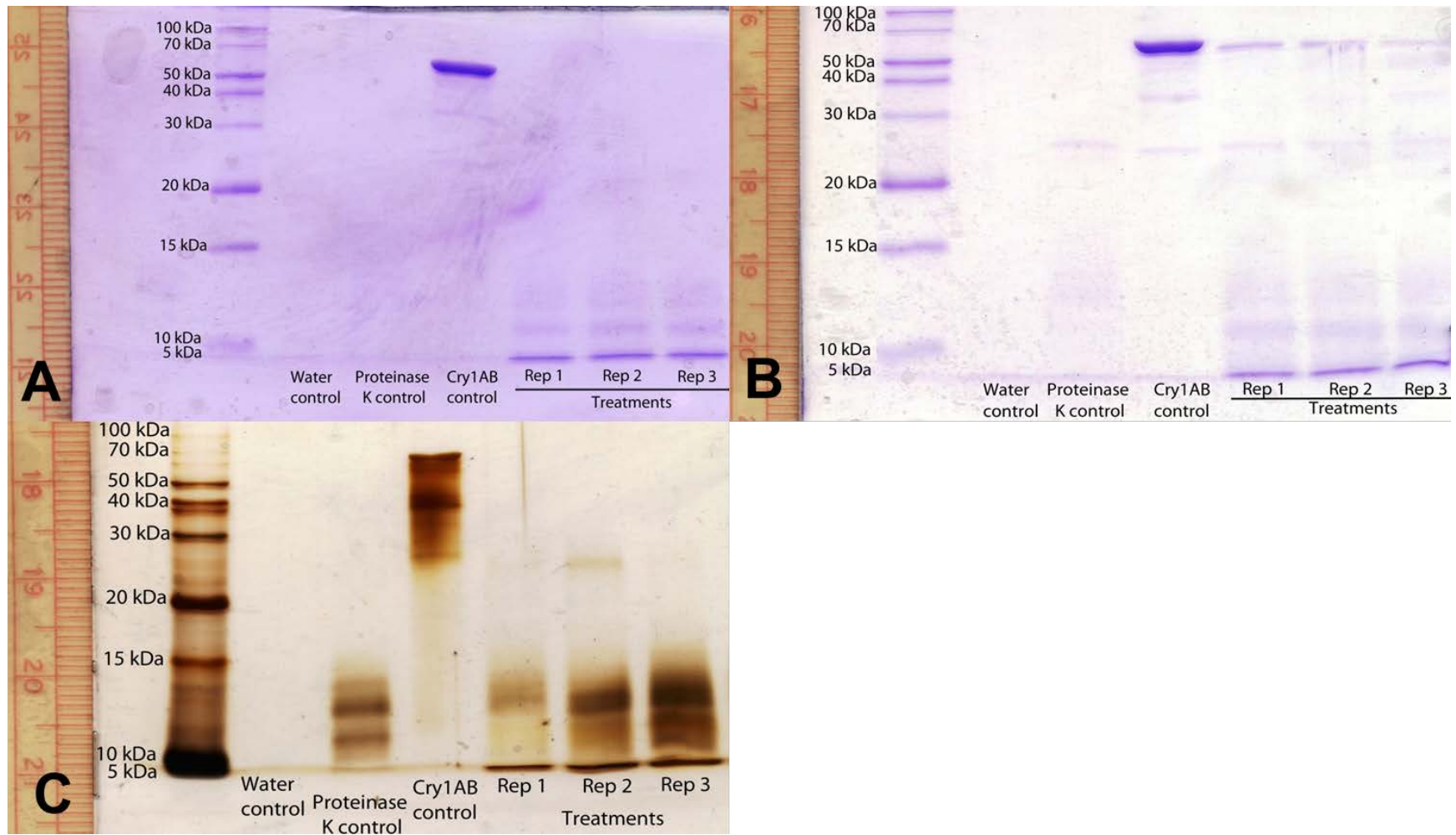


**Figure 7.** Degradation of Cry1Ab with the acidic buffer model system. Incubation times: **A** – 4-5 days, **B** – 6-7 days.

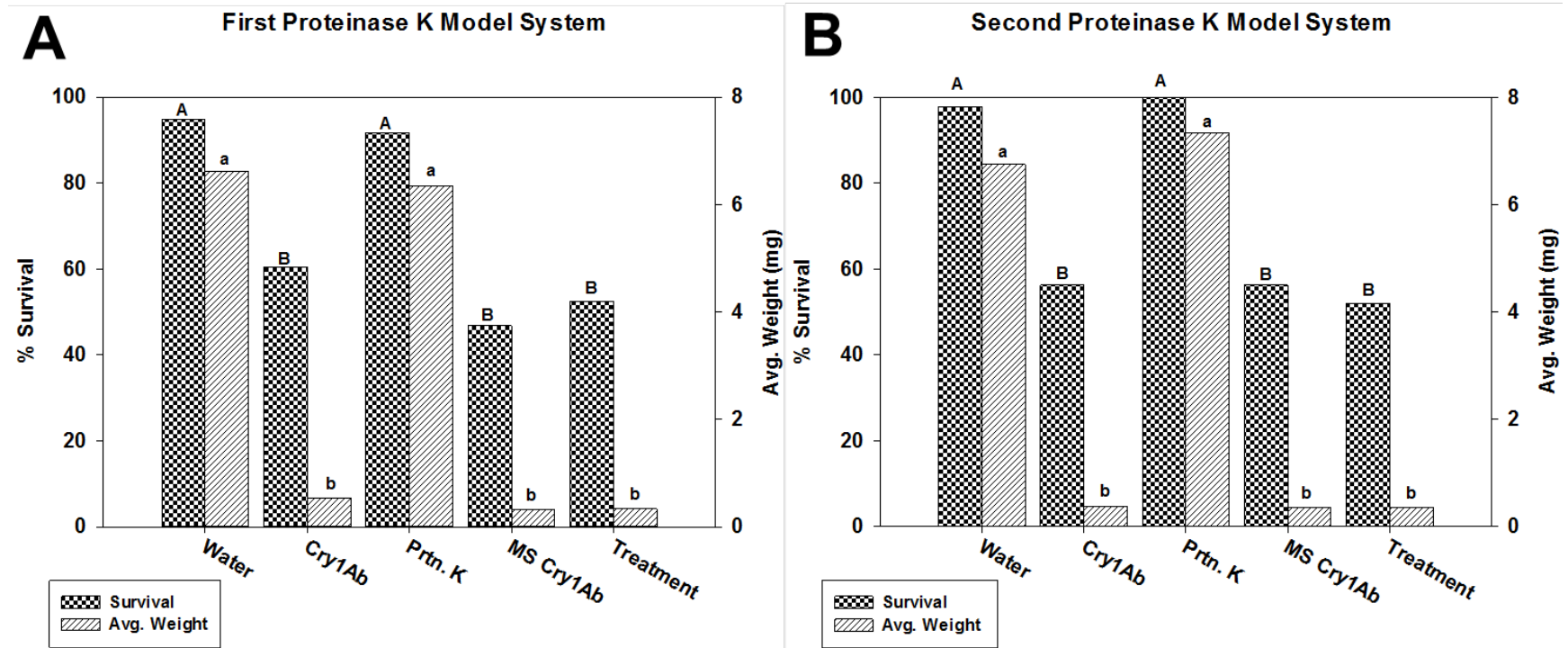


**Figure 8.** **A** – Survival and average weight of surviving insects fed on diet prepared from the 4-5 day acidic buffer model systems. **B** – Survival and average weight of surviving insects fed on diet prepared from the 6-7 day acidic buffer model systems. Cry1Ab = Cry1Ab only; 4 D RT = Cry1Ab incubated in acidic buffer (pH 1.64) at room temperature for 4 days. 4 D Treat = Cry1Ab incubated in acidic buffer (pH 1.64) at 40°C for 4 days. 5 D RT = Cry1Ab incubated in acidic buffer (pH 1.64) at room temperature for 5 days. 5 D Treat = Cry1Ab incubated in acidic buffer (pH 1.64) at 40°C for 5 days. 6 D RT = Cry1Ab incubated in acidic buffer (pH 1.64) at room temperature for 6 days. 6 D Treat = Cry1Ab incubated in acidic buffer (pH 1.64) at 40°C for 6 days. 7 D RT = Cry1Ab incubated in acidic buffer (pH 1.64) at room temperature for 7 days. 7 D Treat = Cry1Ab incubated in acidic buffer (pH 1.64) at 40°C for 7 days. Uppercase letters indicate significance within survival across treatments ( $p = 0.05$ ). Lowercase letters indicate significance within average weight across treatments ( $p = 0.05$ ).



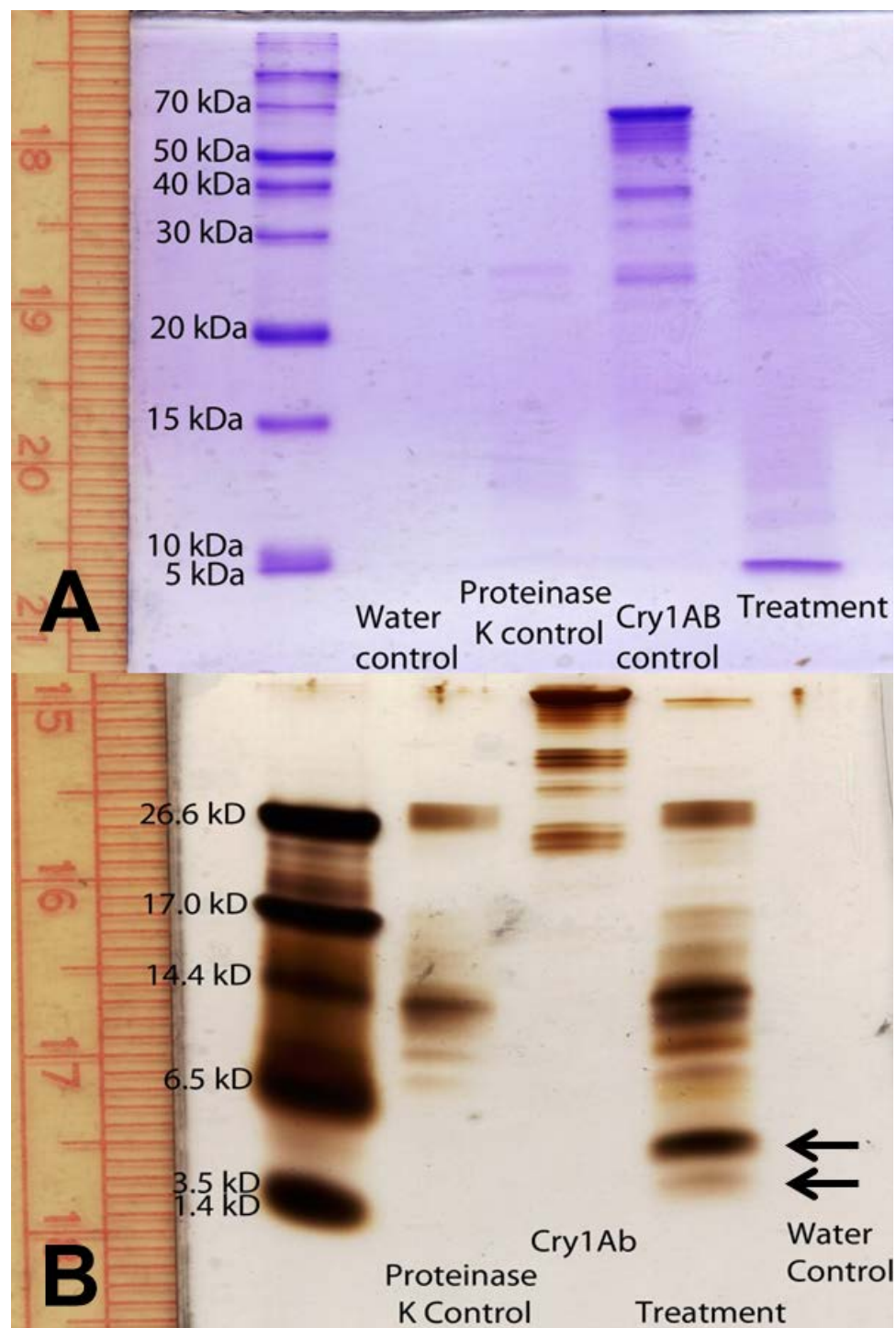


**Figure 9.** Degradation of Cry1Ab with the proteinase K model system. **A** – The proteinase K model system completely degraded Cry1Ab. **B** – The model system was repeated entirely, and the results were replicated. **C** – Silver staining performed on the treatments from the first model system run (**A**) did not detect additional fragment bands.

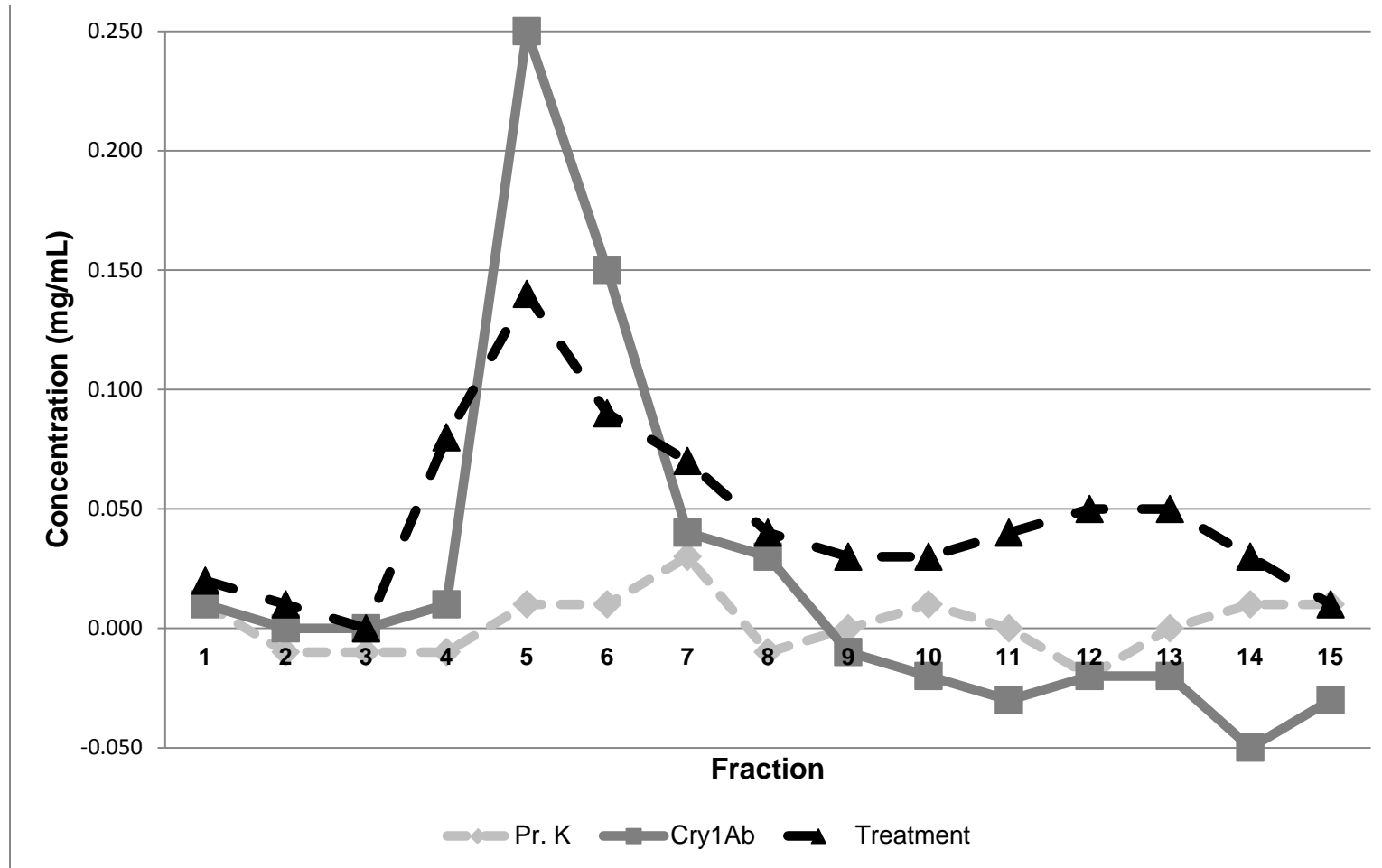


**Figure 10.** Survival and average weight of surviving insects fed on diet prepared from the proteinase K model system. **A** – The initial model system experiment. **B** – The model system experiment was repeated given the interesting survival and average weight results in the treatment. Cry1Ab = Cry1Ab only; Prtn. K = proteinase K in buffer only; MSCry1Ab = Cry1Ab subjected to the model system treatments (37°C for 30 min); Treatment = treatments containing Cry1Ab and proteinase K. Uppercase letters indicate significance within survival across treatments ( $p = 0.05$ ). Lowercase letters indicate significance within average weight across treatments ( $p = 0.05$ ).





**Figure 11.** Mass digest of Cry1Ab by the proteinase K model system. **A** – Fragmentation of the protein was not affected by a mass digest. **B** – Fragments generated by the mass digest were analyzed with a tris-tricine gel and silver stained. Two Cry1ab fragment bands of low molecular weight were identified (indicated by arrows).



**Figure 12.** Graph of results of Sephadex® G-50 separatory column for total protein concentration v. fraction number. Initial sample volume was 3 mL. A majority of the protein in the treatment appears in fractions 4-7, suggesting larger fragments and/or fully intact protein is present. Fractions 4-7 and 12-13 in all treatments were analyzed with ELISAs. Treatment = Cry1Ab degraded with proteinase K; Pr. K = Proteinase K enzyme only; Cry1Ab = Cry1Ab only.

## Tables

**Table 1.** Recipes for separating and stacking gel used in gel electrophoresis.

Separating Gel	Stacking Gel
2.474 mL 30% acrylamide	0.330 mL 30% acrylamide
1.847 mL 1M Tris-HCl, pH 8.8	0.247 mL 1M Tris-HCl, pH 6.8
0.610 mL nanopure water	1.391 mL nanopure water
50 $\mu$ L 10% (w/v) sodium dodecyl sulfate	20 $\mu$ L 10% (w/v) sodium dodecyl sulfate
4 $\mu$ L N,N,N',N'-tetramethylethylenediamine	2 $\mu$ L N,N,N',N'-tetramethylethylenediamine
17 $\mu$ L 10% (w/v) ammonium persulfate	10 $\mu$ L 10% (w/v) ammonium persulfate

**Table 2.** Layout for chymotrypsin model system. At the start of the experiment, the solutions in the initiation column were combined in a vial. All samples were incubated at 37°C for 1 or 24 hr. At the end of the experiment, the solution in the termination column was added. All samples were flash frozen in liquid nitrogen and stored at -80°C until analysis. Cry1Ab = 900 ug/mL Cry1Ab in 50 mM CAPS, pH 10.5; KH<sub>2</sub>PO<sub>4</sub> = 100 mM KH<sub>2</sub>PO<sub>4</sub> pH 7; CAPS = 50 mM CAPS, pH 10.5.

Samples	Initiation	Termination
Cry1Ab Control	75 µL Cry1Ab solution 75 µL KH <sub>2</sub> PO <sub>4</sub>	150 µL water
Chymotrypsin control	75 µL CAPS 75 µL chymotrypsin solution in KH <sub>2</sub> PO <sub>4</sub>	150 µL water
Treatment (in triplicate)	75 µL Cry1Ab solution 75 µL chymotrypsin solution in KH <sub>2</sub> PO <sub>4</sub>	150 µL water

**Table 3.** Layout for proteinase K model system. At the start of the experiment, the solutions in the initiation column were combined in a vial. All samples were incubated at 37°C for 30 min. At the end of the experiment, the solution in the termination column was added. All samples were flash frozen in liquid nitrogen and stored at -80°C until analysis. Cry1Ab = 900 ug/mL Cry1Ab in 50 mM CAPS, pH 10.5; Tris-HCl = 20 mM Tris-HCl, pH 7.5; CAPS = 50 mM CAPS, pH 10.5.

<b>Samples</b>	<b>Initiation</b>	<b>Termination</b>
Water Control	150 µL water	150 µL water
Proteinase K Control	75 µL CAPS 75 µL proteinase K solution in Tris-HCl	150 µL water
Cry1Ab Control	75 µL Tris-HCl 75 µL Cry1Ab solution	150 µL water
Treatment	75 µL Cry1Ab solution 75 µL proteinase K solution in Tris-HCl	150 µL water

**Table 4.** ELISA results of the fractions from the G-50 separatory column experiment. Treatment = Cry1Ab degraded with proteinase K; Pr. K = Proteinase K enzyme only; Cry1Ab = Cry1Ab only. Significant amounts of protein are still detectable in the treatments that have been degraded by proteinase K.

Fraction	Absorbance	Concentration (ng/mL)	Adjusted Concentration (ng/mL)
Treatment			
4	0.299	1.59	11284
5	0.330	2.00	24863
6	0.493	4.19	33654
7	0.232	0.68	4245
12	0.000	0.00	0
13	0.006	0.00	0
Pr. K			
4	0.008	0.00	0
5	-0.004	0.00	0
6	-0.004	0.00	0
7	-0.005	0.00	0
12	-0.005	0.00	0
13	0.019	0.00	0
Cry1Ab			
4	0.007	0.00	0
5	0.354	2.33	51792
6	0.840	8.87	118051
7	0.917	9.92	35265
12	0.050	0.00	0
13	0.028	0.00	0

**Table 5.** Relative percent difference (R%D) values for the standard curve prepared in chymotrypsin model system buffer and the standard curve prepared in assay buffer (PBST). Shaded cells fall outside the acceptance criterion of  $R\%D \leq |15\%|$  between the assay buffer and model system buffer. If the  $R\%D > |15\%|$  across two consecutive points in the standard curve, then there may be matrix effects due to the model system components. No buffer or matrix effects are expected to occur in a 1:15,000 or a 1:22,500 dilution.

Standard Curve	Dilution factor (1:x)			
	15,000	20,000	22,500	50,000
20 ng/mL	-8.28	-5.91	-23.75	-12.98
16 ng/mL	-1.00	11.32	-13.15	-14.32
12 ng/mL	-8.01	-9.37	-14.70	-25.83
8 ng/mL	0.04	-25.60	1.04	-34.50
4 ng/mL	-8.10	-30.30	-20.16	-37.85
1 ng/mL	-1.71	-28.48	-12.85	-22.27

**Table 6.** Percent coefficient of variation (%CV) for the chymotrypsin model system buffer dilutions. Shaded cells in the upper portion of the table fall outside the quantitative range, or were purposely excluded to bring the %CV value in range. Shaded cells in the lower portion of the table fall outside the acceptance criterion of  $\%CV \leq 20\%$ . For those cells with %CV outside of the acceptance criterion, minimum or maximum dilutions were applied until the %CV was  $\leq 20\%$ . Dilution the samples by a factor of 1:15,000 – 1:50,000 is not expected to affect protein quantitation.

	Concentration		Mean Result (ng/mL)	Adjusted Result (ng/mL)	Removing out of range dilutions	Applying maximum dilution of 1:50000
	Rep 1	Rep 2				
1:5,000	15.15	14.69	14.92	74587	Above ULOQ	Above ULOQ
1:10,000	8.67	9.43	9.05	90528	Above ULOQ	Above ULOQ
1:15,000	4.49	7.92	6.20	93069	93069	93069
1:20,000	3.47	5.01	4.24	84851	84851	84851
1:22,500	3.67	5.49	4.58	103022	103022	103022
1:50,000	0.36	3.49	1.93	96254	96254	96254
1:100,000	-1.06	-1.08	-1.07	-106724	-106724	Excluded
1:1,000,000	-3.01	-3.04	-3.02	-3022094	Below LLOQ	Below LLOQ
Mean adjusted result				-323313	54094	94299
Standard deviation				1092683	90137	7543
%CV				-337.96	166.63	8.00



**Table 7.** ELISA results from the chymotrypsin model system. Adjusted concentration was calculated by multiplying by the dilution factor, 1:22,500. % Cry1Ab remaining was calculated by dividing the concentration each of the replicates by the Cry1Ab control concentration. ELISA results indicate little degradation has occurred at 1 hour, and significant degradation has occurred at 24 hours.

1 hour	Absorbance	Concentration (ng/mL)	Adjusted Concentration (ng/mL)	% Cry1Ab Remaining
Cry1Ab control	0.466	4.22	95000	
Chymo control	0.034	-2.83	0	
Replicate 1	0.493	4.66	104804	110.3%
Replicate 2	0.456	4.06	91324	96.13
Replicate 3	0.456	4.05	91160	95.96
24 hour	Absorbance	Concentration (ng/mL)	Adjusted Concentration (ng/mL)	% Cry1Ab Remaining
Cry1Ab control	0.338	2.14	48105	
Chymo control	0.008	-3.27	0	
Replicate 1	0.258	0.83	18652	38.77
Replicate 2	0.296	1.45	32663	67.90
Replicate 3	0.235	0.45	10155	21.11

**Table 8.** Relative percent difference (R%D) values for the standard curve prepared in CAPS buffer and water and the standard curve prepared in assay buffer (PBST). This CAPS/water buffer system was utilized in both the long-term degradation in buffer model system and the photodegradation model system. Shaded cells fall outside the acceptance criterion of  $R\%D \leq |15\%|$  between the assay buffer and model system buffer. If the  $R\%D > |15\%|$  across two consecutive points in the standard curve, then there may be matrix effects due to the model system components. No buffer or matrix effects are expected to occur across a dilution range of 1:10,000 – 1:50,000.

Standard Curve	Dilution factor (1:x)			
	10,000	50,000	10,0000	1,000,000
20 ng/mL	-9.06	-9.12	-14.19	-13.32
16 ng/mL	-9.28	-7.36	-14.95	-8.92
12 ng/mL	-3.94	-6.52	-17.53	-22.67
8 ng/mL	-15.54	-14.12	-17.84	-16.56
4 ng/mL	-3.96	-14.43	-16.44	-20.54
1 ng/mL	-12.30	-12.57	-16.58	-28.88

**Table 9.** Percent coefficient of variation (%CV) for CAPS buffer and water dilutions. This CAPS/water buffer system was utilized in both the long-term degradation in buffer model system and the photodegradation model system. Shaded cells in the upper portion of the table fall outside the quantitative range, or were purposely excluded to bring the %CV value in range. Shaded cells in the lower portion of the table fall outside the acceptance criterion of  $\%CV \leq 20\%$ . For those cells with %CV outside of the acceptance criterion, minimum or maximum dilutions were applied until the %CV was  $\leq 20\%$ . Diluting the samples by a factor of 1:15,000 – 1:50,000 is not expected to affect protein quantitation.

	Concentration		Mean Result (ng/mL)	Adjusted Result (ng/mL)	Removing out of range dilutions	Applying maximum dilution of 1:50000
	Rep 1	Rep 2				
1:5,000	22.88	23.19	23.03	115161	Above ULOQ	Above ULOQ
1:10,000	18.55	17.76	18.15	181523	Above ULOQ	Above ULOQ
1:15,000	12.87	12.90	12.89	193292	193292	193292
1:20,000	9.55	10.36	9.96	199127	199127	199127
1:22,500	8.29	8.76	8.53	191819	191819	191819
1:50,000	3.77	4.75	4.26	213024	213024	213024
1:100,000	0.74	0.92	0.83	83333	83333	Excluded
1:1,000,000	-1.99	-0.50	-1.24	-1241274	Below LLOQ	Below LLOQ
Mean adjusted result				-7999	176119	199316
Standard deviation				500370	52540	9668
%CV				-6255.18	29.83	4.85

**Table 10.** ELISA results from the long-term degradation in buffer model system.

Adjusted concentration was calculated by multiplying by the dilution factor, 1:22,500. % Cry1Ab remaining was calculated by dividing the concentration each of the replicates by the Cry1Ab control concentration. ELISA results indicate significant degradation has occurred after 16 weeks.

	Absorbance	Concentration (ng/mL)	Adjusted Concentration (ng/mL)	% Cry1Ab Remaining
Cry Control	0.553	5.75	129333	
Buffer Control	0.002	-2.12	0	
Replicate 1	0.354	2.90	65317	50.50
Replicate 2	0.362	3.01	67743	52.38
Replicate 3	0.349	2.83	63570	49.15

**Table 11.** ELISA results from the photodegradation model system. Adjusted concentration was calculated by multiplying by the dilution factor, 1:22,500. % Cry1Ab remaining was calculated by dividing the concentration each of the replicates by the Cry1Ab control concentration. ELISA results indicate significant degradation has occurred at both time points.

	Absorbance	Concentration (ng/mL)	Adjusted Concentration (ng/mL)	% Cry1Ab Remaining
Cry1Ab Control	0.636	6.04	136003	
Buffer Control	-0.002	-1.78	0	
2.5 minutes				
Replicate 1	0.144	0.00	0	0.00
Replicate 2	0.156	0.15	3457	2.54
Replicate 3	0.033	-1.35	0	0.00
5 minutes				
Replicate 1	0.062	-1.01	0	0.00
Replicate 2	0.027	-1.43	0	0.00
Replicate 3	0.134	-0.11	0	0.00

**Table 12.** Relative percent difference (R%D) values for the standard curve prepared in acidic buffer and the standard curve prepared in assay buffer (PBST). Shaded cells fall outside the acceptance criterion of  $R\%D \leq |15\%|$  between the assay buffer and model system buffer. If the  $R\%D > |15\%|$  across two consecutive points in the standard curve, then there may be matrix effects due to the model system components. No buffer or matrix effects are expected to occur across a dilution range of 1:10,000 – 1:1,000,000.

	Dilution factor (1:x)			
Standard Curve	10,000	50,000	100,000	1,000,000
20 ng/mL	4.18	3.36	-12.28	9.33
16 ng/mL	-10.83	-16.01	26.78	-4.79
12 ng/mL	11.21	7.62	-3.64	-13.55
8 ng/mL	7.72	4.33	3.48	-10.35
4 ng/mL	-18.78	-3.32	4.17	-1.14
1 ng/mL	-10.87	-25.15	-28.17	-15.11

**Table 13.** Percent coefficient of variation (%CV) for acidic buffer model system dilutions. Shaded cells in the upper portion of the table fall outside the quantitative range, or were purposely excluded to bring the %CV value in range. Shaded cells in the lower portion of the table fall outside the acceptance criterion of  $\%CV \leq 20\%$ . For those cells with %CV outside of the acceptance criterion, minimum or maximum dilutions were applied until the %CV was  $\leq 20\%$ . Diluting the samples by a factor of 1:15,000 – 1:100,000 is not expected to affect protein quantitation.

	Concentration		Mean Result (ng/mL)	Adjusted Result (ng/mL)	Removing out of range dilutions
	Rep 1	Rep 2			
1:5,000	19.55	19.57	19.56	97795	Above ULOQ
1:10,000	15.40	14.83	15.12	151151	Above ULOQ
1:15,000	11.84	12.22	12.03	180419	180419
1:20,000	9.28	8.51	8.90	177922	177922
1:22,500	7.65	7.84	7.74	174262	174262
1:50,000	3.27	3.55	3.41	170581	170581
1:100,000	1.02	1.12	1.07	107054	107054
1:1,000,000	-1.42	-1.42	-1.42	-1421705	Below LLOQ
Mean adjusted result				-45315	162048
Standard deviation				557079	30967
%CV				-1229.34	19.11

**Table 14.** ELISA results from the acidic buffer model system. Adjusted concentration was calculated by multiplying by the dilution factor, 1:22,500. % Cry1Ab remaining was calculated by dividing the concentration each of the replicates by the Cry1Ab control concentration. ELISA results indicate significant degradation has occurred in all treatments and controls at all time points.

	Absorbance	Concentration (ng/mL)	Adjusted Concentration (ng/mL)	% Cry1Ab Remaining
Cry1Ab control	0.713	5.42	121909	
Acidic buffer ctrl	0.007	-2.01	0	
Day 4				
Room Temp.	0.003	-2.05	0	0
Replicate 1	0.002	-2.06	0	0
Replicate 2	0.010	-1.98	0	0
Day 5				
Room Temp.	0.011	-1.97	0	0
Replicate 1	0.009	-1.99	0	0
Replicate 2	0.004	-2.04	0	0
Day 6				
Room Temp.	0.175	-0.24	0	0
Replicate 1	0.011	-1.97	0	0
Replicate 2	0.007	-2.00	0	0
Day 7				
Room Temp.	0.006	-2.01	0	0
Replicate 1	0.012	-1.95	0	0
Replicate 2	0.013	-1.94	0	0



**Table 15.** Relative percent difference (R%D) values for the standard curve prepared in proteinase K model system buffer and the standard curve prepared in assay buffer (PBST). Shaded cells fall outside the acceptance criterion of  $R\%D \leq |15\%|$  between the assay buffer and model system buffer. If the  $R\%D > |15\%|$  across two consecutive points in the standard curve, then there may be matrix effects due to the model system components. No buffer or matrix effects are expected to occur across a dilution range of 1:10,000 – 1:50,000.

	Dilution factor (1:x)				
Standard Curve	10,000	15,000	20,000	22,500	50,000
20 ng/mL	-3.74	-14.18	-8.70	-11.17	-12.98
16 ng/mL	7.17	-24.66	7.54	-6.33	6.33
12 ng/mL	17.21	5.26	-1.21	-0.27	12.10
8 ng/mL	2.57	11.07	-7.64	-4.38	-5.36
4 ng/mL	5.70	14.08	13.70	19.56	-7.89
1 ng/mL	8.49	-1.04	-0.41	-11.59	-14.29

**Table 16.** Percent coefficient of variation (%CV) for the proteinase K model system buffer dilutions. Shaded cells in the upper portion of the table fall outside the quantitative range, or were purposely excluded to bring the %CV value in range. Shaded cells in the lower portion of the table fall outside the acceptance criterion of  $\%CV \leq 20\%$ . For those cells with %CV outside of the acceptance criterion, minimum or maximum dilutions were applied until the %CV was  $\leq 20\%$ . Diluting the samples by a factor of 1:15,000 – 1:50,000 is not expected to affect protein quantitation.

	Concentration		Mean Result (ng/mL)	Adjusted Result (ng/mL)	Removing out of range dilutions	Applying maximum dilution of 1:50000
	Rep 1	Rep 2				
1:5,000	22.34	20.48	21.41	107057	Above ULOQ	Above ULOQ
1:10,000	17.76	18.41	18.08	180819	Above ULOQ	Above ULOQ
1:15,000	14.23	13.71	13.97	209521	209521	209521
1:20,000	10.57	10.12	10.35	206928	206928	206928
1:22,500	9.48	8.62	9.05	203599	203599	203599
1:50,000	3.07	3.46	3.27	163418	163418	163418
1:100,000	0.84	0.48	0.66	65782	65782	Excluded
1:1,000,000	-1.84	-1.90	-1.87	-1868128	Below LLOQ	Below LLOQ
Mean adjusted result				-91375	169850	195866
Standard deviation				719768	61154	21768
%CV				-787.70	36.00	11.11

**Table 17.** ELISA results from the proteinase K model system. Adjusted concentration was calculated by multiplying by the dilution factor, 1:20,000. % Cry1Ab remaining was calculated by dividing the concentration each of the replicates by the Cry1Ab control concentration. **A** – ELISA results from the first model system run indicate little degradation has occurred. **B** – ELISA results from the second model system run also indicate little degradation has occurred.

<b>A</b>	Absorbance	Concentration (ng/mL)	Adjusted Concentration (ng/mL)	% Cry1Ab Remaining
Cry1Ab control	0.726	5.15	102930	
Pr. K control	0.006	-2.73	0	
Replicate 1	0.719	5.07	101471	98.58
Replicate 2	0.690	4.75	95077	92.37
Replicate 3	0.546	3.17	63469	62.55
<b>B</b>	Absorbance	Concentration (ng/mL)	Adjusted Concentration (ng/mL)	% Cry1Ab Remaining
Cry1Ab control	0.713	5.62	112406	
Pr. K control	-0.021	-2.93	0	
Replicate 1	0.695	5.41	108262	96.31
Replicate 2	0.606	4.37	87464	77.81
Replicate 3	0.632	4.67	93370	83.06

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## CHAPTER 4. GENERAL CONCLUSIONS

The purpose of this research was to further the knowledge of the fate of Cry proteins in the environment. Specifically, this dissertation sought to address the question of whether fragments of Cry proteins are detectable by enzyme-linked immunosorbent assays (ELISAs), which are the preferred method of detection for Cry proteins in the environment. If Cry protein fragments are detectable by ELISAs, then biological validation of ELISA results may be necessary to characterize biological activity in environmental samples. There were two main objectives to accomplish this goal. For the first objective, numerous model systems were screened for their ability to generate fragments of the Cry1Ab protein in a controlled and predictable manner. For the second objective, these fragments were analyzed with bioassays and ELISAs to determine if these fragments retained bioactivity and if they were still detectable by ELISA.

The screening of multiple model systems for their capability to generate fragments of Cry1Ab was investigated in Chapter 2. Eight different types of model systems were screened. The trypsin model system and the simulated gut fluid model system did not sufficiently degrade Cry1Ab, while the pepsin model system completely degraded the protein, but did not produce any protein fragments detectable by gel electrophoresis. The remaining five model systems yielded various types and quantities of Cry1Ab fragments. The fragments from these model systems, the chymotrypsin model system, proteinase K model system, acidic buffer model system, photodegradation model system, and the long-term degradation in buffer model system, were then used in the second objective (Chapter 3).

In Chapter 3, the model systems identified in Chapter 2 were used to generate fragments that were then analyzed with ELISAs and bioassays to determine if the fragments were still detectable and if they retained any bioactivity. The results indicated that fragments generated by four of the model systems were not detectable by ELISA and did not retain any bioactivity. These four model systems were the chymotrypsin model system, acidic buffer model system, photodegradation model system, and long-term degradation in buffer model system. Despite being highly degraded, the fragments generated by the proteinase K model system were still detectable by ELISA and still retained biological activity. Further investigations suggest that these fragments may be associating with each other, but more research is needed to understand this phenomenon.

The primary concern at the beginning of this research was that fragments of Cry proteins may still be detectable by ELISAs, but not retain any biological activity. If true, this could lead to an over-estimation of the amount of biologically active protein in the environment, which in turn could affect the risk assessments for these proteins. The research described here primarily indicated that this was not observed in the model systems tested in this research project. Cry1Ab fragments that were detectable by ELISA, also retained bioactivity; those fragments that were not detectable, did not have bioactivity. Therefore, researchers using ELISA kits appear to be capturing an accurate picture of the amount of Cry protein in environmental matrices. However, more research is needed in this area to test additional ELISA kits and other model systems, including microbe-based model systems, in order to better understand the fate of Cry proteins in the environment.

## APPENDIX A: CHAPTER 2 SUPPLEMENTAL INFORMATION

In addition to the buffers and inhibitors tested in Chapter 2, other buffers and inhibitors were tested that produced unsatisfactory results.

### *Trypsin*

The trypsin model system was originally screened with a Tris-HCl buffer (0.1 M Tris-HCl, 0.15 M sodium chloride, 5 mM magnesium chloride, pH 7.5), and using an equal volume of 20% (w/v) trichloroacetic acid as an inhibitor. An initial bioassay test showed that the trichloroacetic acid was acutely toxic to the insects, and its use in the model system was quickly abandoned (Figure 1). Trypsin inhibitor from *Glycine max* was purchased, and another screening bioassay was performed. The Tris-HCl buffer described above did not affect insect survival, but significantly reduced larval weights (Figure 2). This buffer also was abandoned, and the seven buffers described in Chapter 2 were screened for use.

### *Pepsin*

The pepsin model system was initially tested using sodium bicarbonate as an inhibitor. An activity assay, similar to the one described in Chapter 2, determined that complete inhibition of pepsin could be achieved at 10% (w/v) sodium bicarbonate (Figure 3). However, the presence of sodium bicarbonate caused significant mortality in the bioassays, and its use as an inhibitor was discontinued (Figure 4). Sodium hydroxide was then chosen as the inhibitor and was used as described in Chapter 2.



*Proteinase K*

An inhibitor was originally included in the proteinase K model system, but was removed due to high background absorbance in the activity assay. A known substrate for proteinase K, acetyl-L-tyrosine ethyl ester, was not available at the time, so the feasibility of using N-benzoyl-L-tyrosine ethyl ester (BTEE) was investigated. This was deemed an acceptable substitute as proteinase K cleaves adjacent to the carboxylic group in aromatic amino acids (Ebeling *et al.* 1974). A 5-mM concentration of BTEE was prepared by dissolving the substrate in 50% (v/v) methanol. One milliliter of this solution was combined with 4 mL of four different proteinase K solutions (5, 25, 100, and 500 µg/mL proteinase K). The background absorbance of proteinase K and BTEE only was 0.041 and 2.251, respectively. The absorbance in the proteinase K solutions was 2.377-2.815. Since the background absorbance of proteinase K plus the background absorbance of BTEE (2.292) was less than then the absorbance observed in the samples, the increased absorbance in the samples must have come from proteinase K cleaving BTEE; this information supported using BTEE as a substrate for proteinase K. An activity assay was then set up using 5 mM phenylmethane sulfonyl fluoride (PMSF) as the inhibitor. The background absorbance values for proteinase K, BTEE, and PMSF were 0.199, 2.227, and 1.429, respectively. When combined, these gave a total background of 3.855, which exceeds the maximum absorbance recordable, and leaves no margin for an increase in absorbance due to cleavage of the BTEE substrate. Instead of working with the activity assay further, the decision was made to try degrading Cry1Ab first to determine if further studies with proteinase K would be worthwhile. During this experiment (Figure 19, Chapter 2), it was determined that flash freezing the samples in liquid nitrogen was

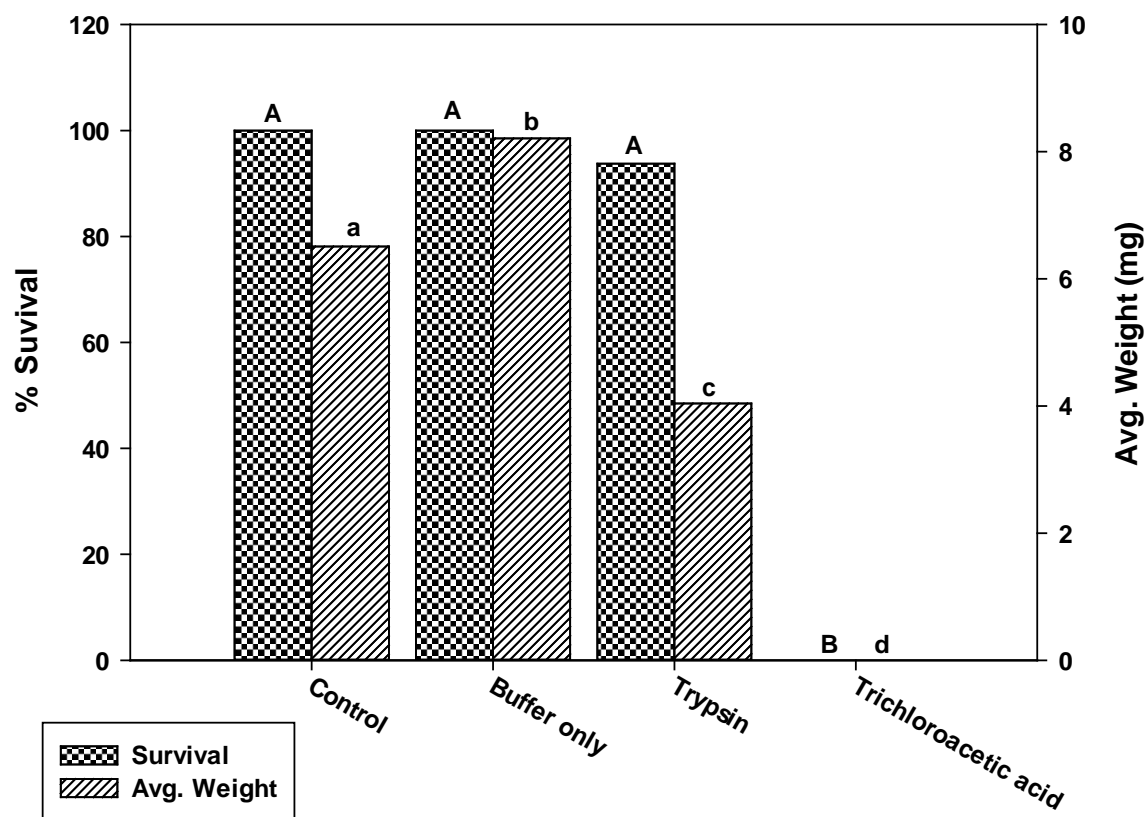
sufficient for termination of the reaction. This termination mechanism also was employed in the chymotrypsin model system without any issues.

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Proteinase K from *Tritirachium album* Limber. *Eur. J. Biochem.* **47**: 91-97.

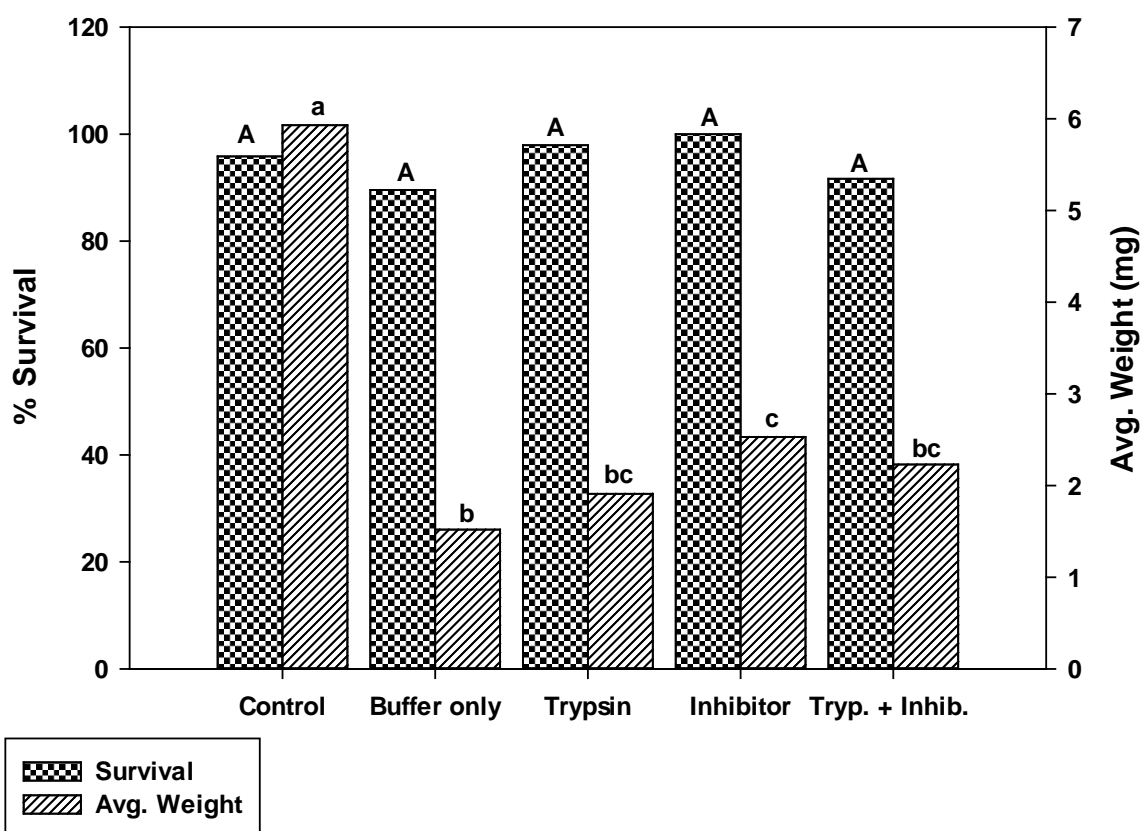
## Figures

## Initial Trypsin Model System Test

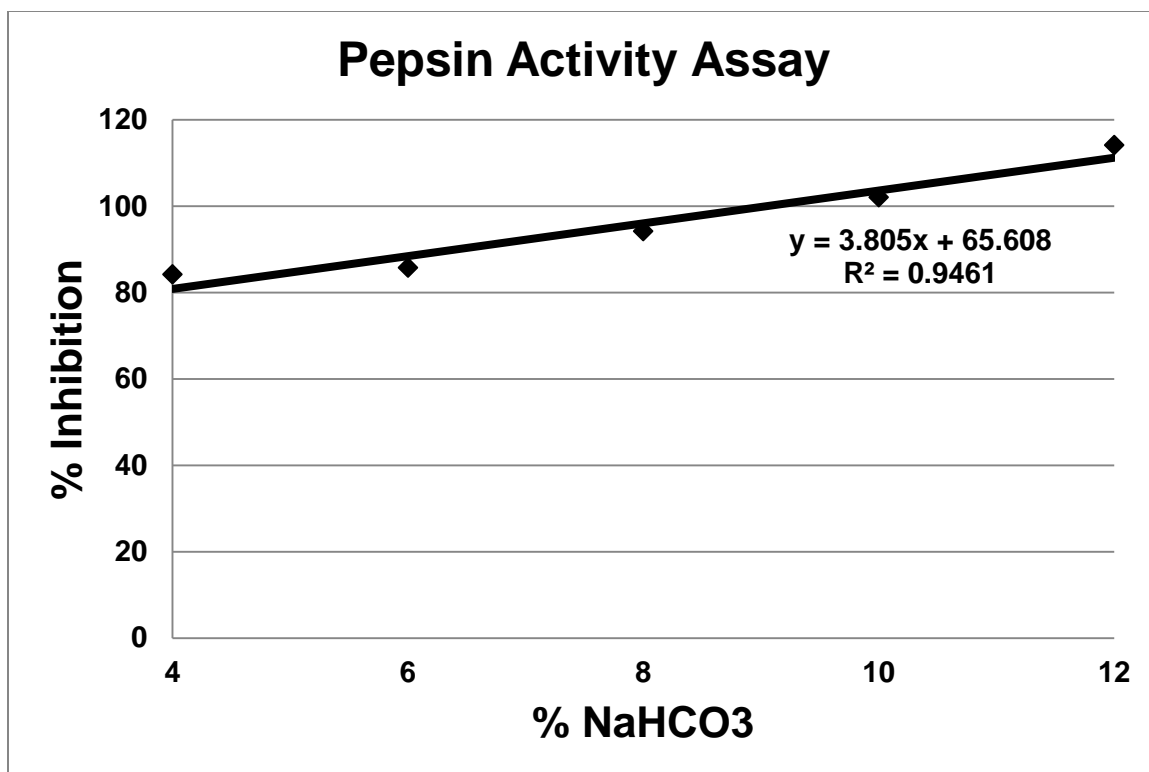


**Figure 1.** Bioassay results from the initial trypsin model system test using a Tris-HCl buffer and trichloroacetic acid as the inhibitor. Uppercase letters indicate significance within survival across buffers ( $p = 0.05$ ). Lowercase letters indicate significance within average weight across buffers ( $p = 0.05$ ).

## Second Trypsin Model System Test

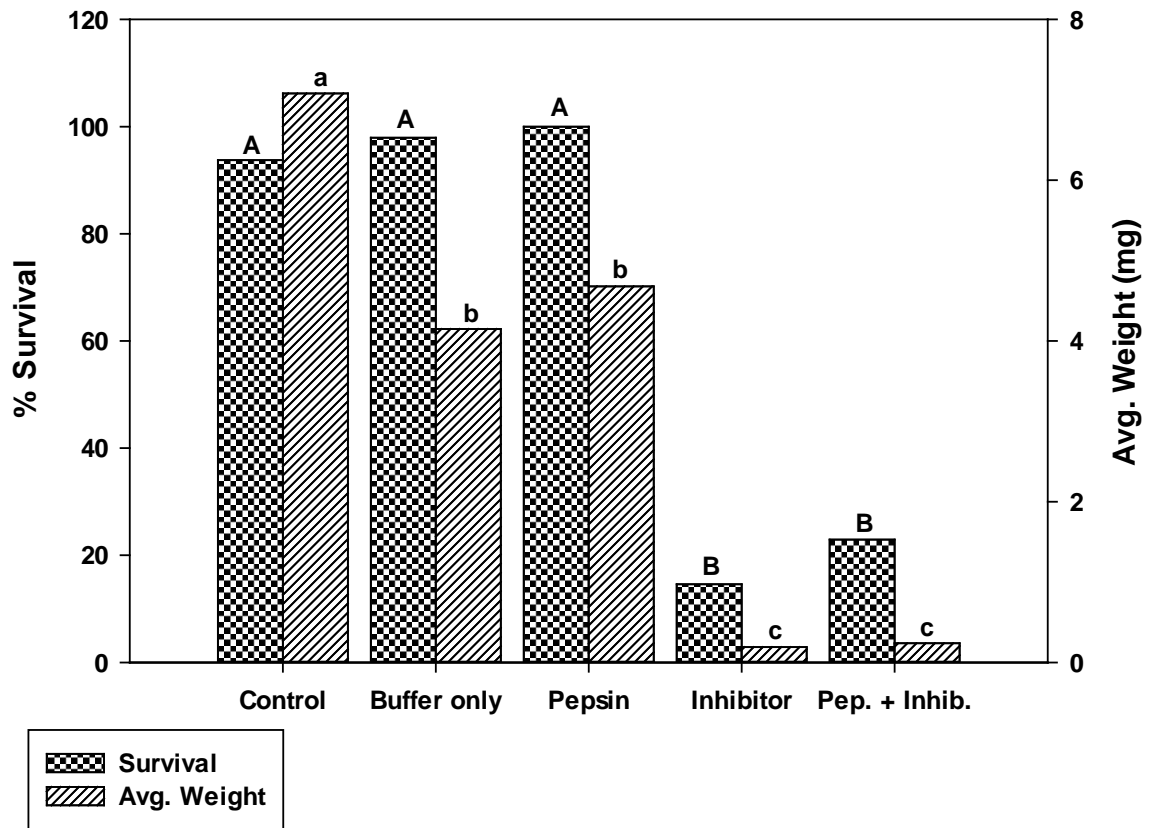


**Figure 2.** Bioassay results from the second trypsin model system test using a Tris-HCl buffer and trypsin inhibitor from *Glycine max*. Uppercase letters indicate significance within survival across buffers ( $p = 0.05$ ). Lowercase letters indicate significance within average weight across buffers ( $p = 0.05$ ).



**Figure 3.** Pepsin activity assay results with sodium bicarbonate as an inhibitor. Complete inhibition is achieved at 10% (w/v) sodium bicarbonate.

### Initial Pepsin Model System Test



**Figure 4.** Bioassay results from the initial pepsin model system test using 10% (w/v) sodium bicarbonate as the inhibitor. Uppercase letters indicate significance within survival across buffers ( $p = 0.05$ ). Lowercase letters indicate significance within average weight across buffers ( $p = 0.05$ ).

## **APPENDIX B: CHAPTER 3 SUPPLEMENTAL INFORMATION**

### *G-50 Separatory Column Experiment*

Bioassays also were performed on the fractions generated by the G-50 separatory column experiment in Chapter 3. Significant challenges were encountered while trying to obtain the bioassay data from this experiment. The water control survival in several bioassay experiments fell below the acceptable threshold (survival <80%). The need to repeat these experiments numerous times led to multiple freeze-thaw cycles, which compromised the integrity of the protein. This compromise was discovered when ELISAs were repeated. The first ELISA run did not contain a fully intact Cry1Ab standard to compare to the results. When a second ELISA was performed on the same fractions, significant losses in detectable protein were observed in the treatment and Cry1Ab control samples (Table 1B). The multiple freeze/thaw cycles the protein solutions were exposed to while trying to complete the bioassay results is probably responsible for the loss of detectable protein. Due to this discrepancy in ELISA data, the bioassay results must be classified as inconclusive. These bioassay results are reported here for reference, and any assumptions made from these results should be considered inconclusive (Figures 1-3).

### *Native PAGE*

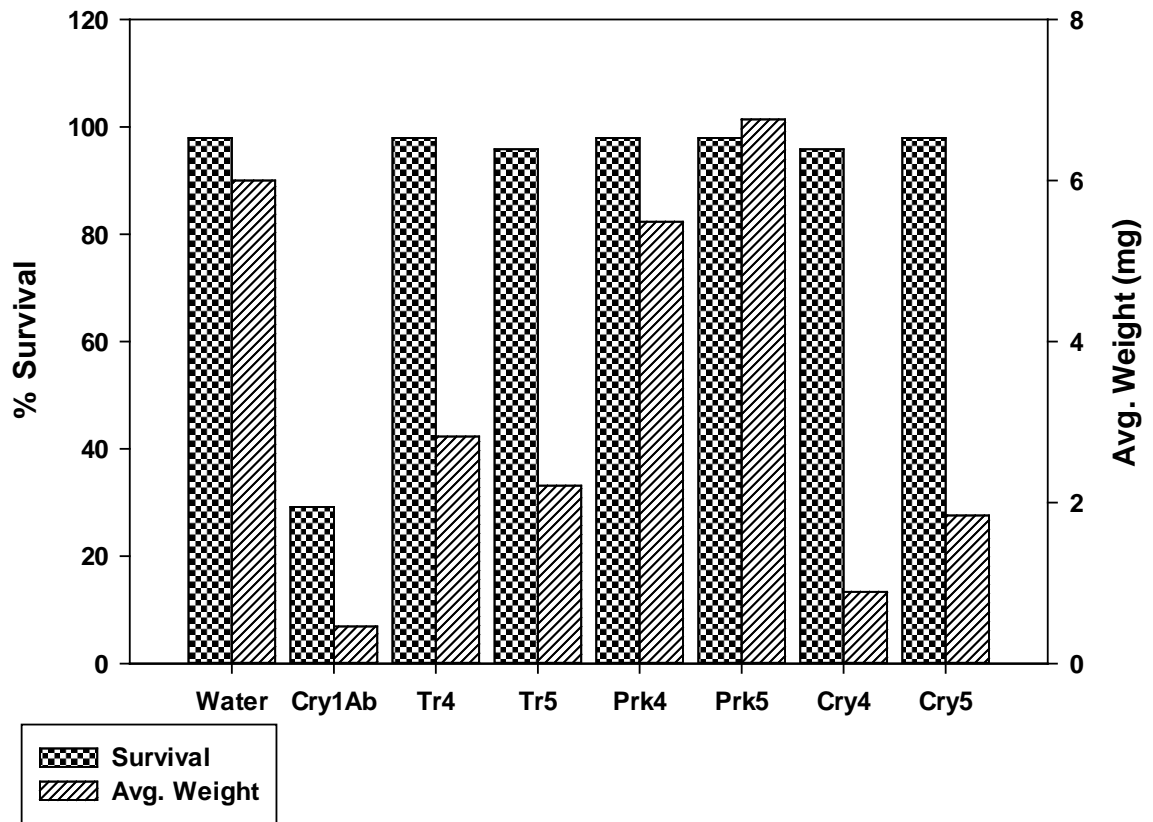
A native PAGE experiment was performed on an aliquot of the proteinase K mass digest. Native PAGE gels do not contain SDS or mercaptoethanol and heating is not used; thus denaturing of the protein should not occur (*i.e.*, disulfide bonds should remain intact) and the protein should retain its native conformation. If the fragments are not

associated together, then, multiple bands should be visible; however, if the fragments are associating to each other, only one band should be visible. Sample buffer, running buffer and gels free of denaturing agents were prepared. Sample buffer consisted of 62.5 mM Tris-HCl, pH 6.8, 25% (w/v) glycerol, and 1% (w/v) bromophenol blue. The running buffer was a 40 mM CAPS buffer, pH 11. An 8% separating gel and the stacking gel were prepared as described in Table 2. Samples were mixed at a 1:1 ratio of sample to sample buffer and allowed to sit for 10 minutes. Samples were centrifuged at 12 x g for 5 minutes and 20 uL of each sample was loaded in each lane. A constant 150 volts was applied to the gel for 30-40 min. The gel is in Figure 4. Only one band appears in the treatment. This band migrated approximately the same distance as the fully-intact Cry1Ab, suggesting the fragments are still associated. It was only after performing this experiment that we learned that native PAGE gel results are dependent on pH and isoelectric point, which can vary significantly depending on gel conditions. Therefore, the reliability of this experiment is questionable and was not included in Chapter 3.



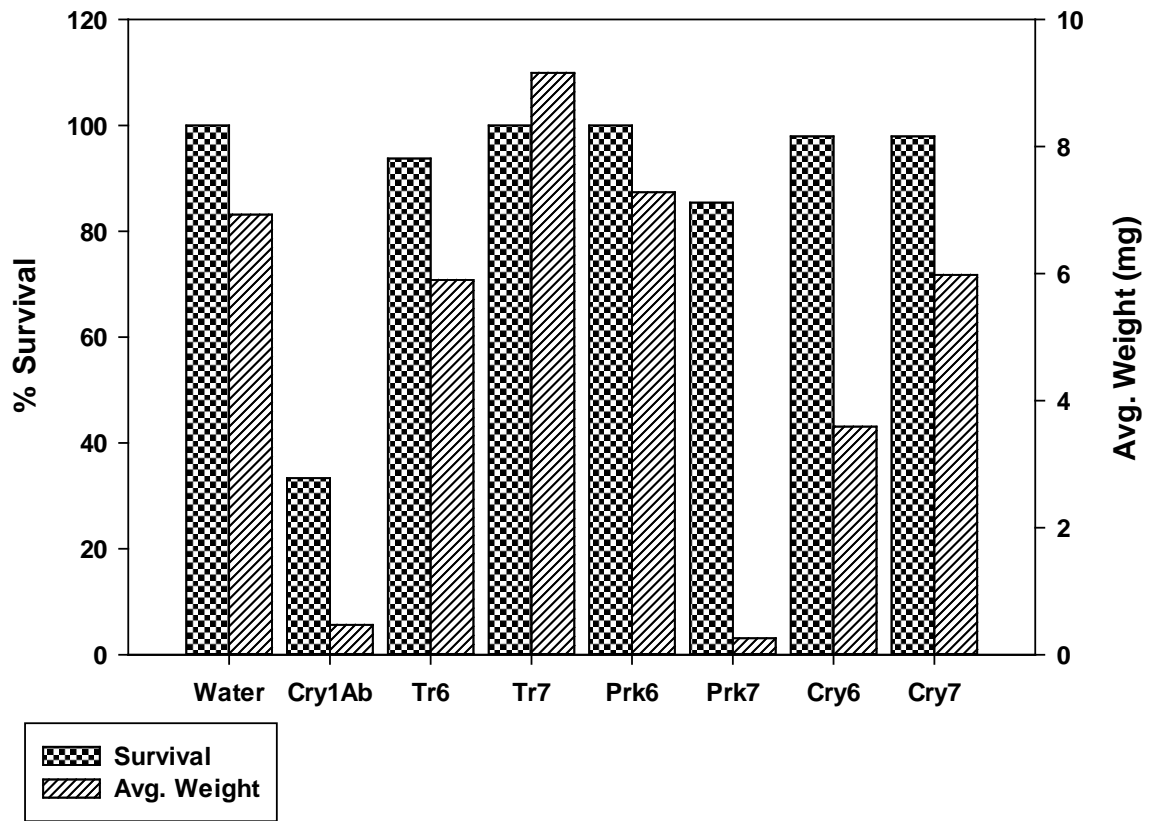
## Figures

### Fractions 4-5 Bioassays



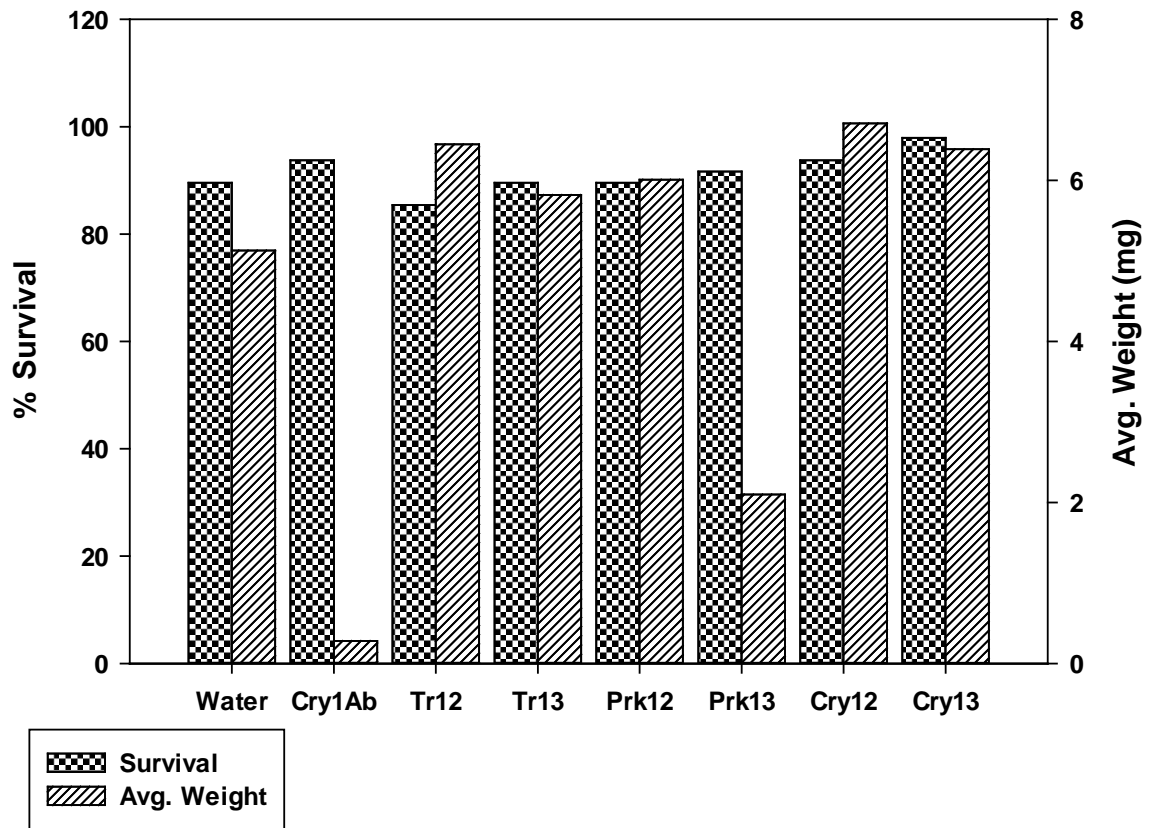
**Figure 1.** Bioassay results from fractions 4-5 of the treatment, Cry1Ab, and proteinase K solutions. Tr = Treatment, Cry1Ab degraded with proteinase K; PrK = Proteinase K enzyme only; Cry = Cry1Ab only. No useful information can be gained from these bioassays, as the solutions were compromised.

### Fraction 6-7 Bioassays

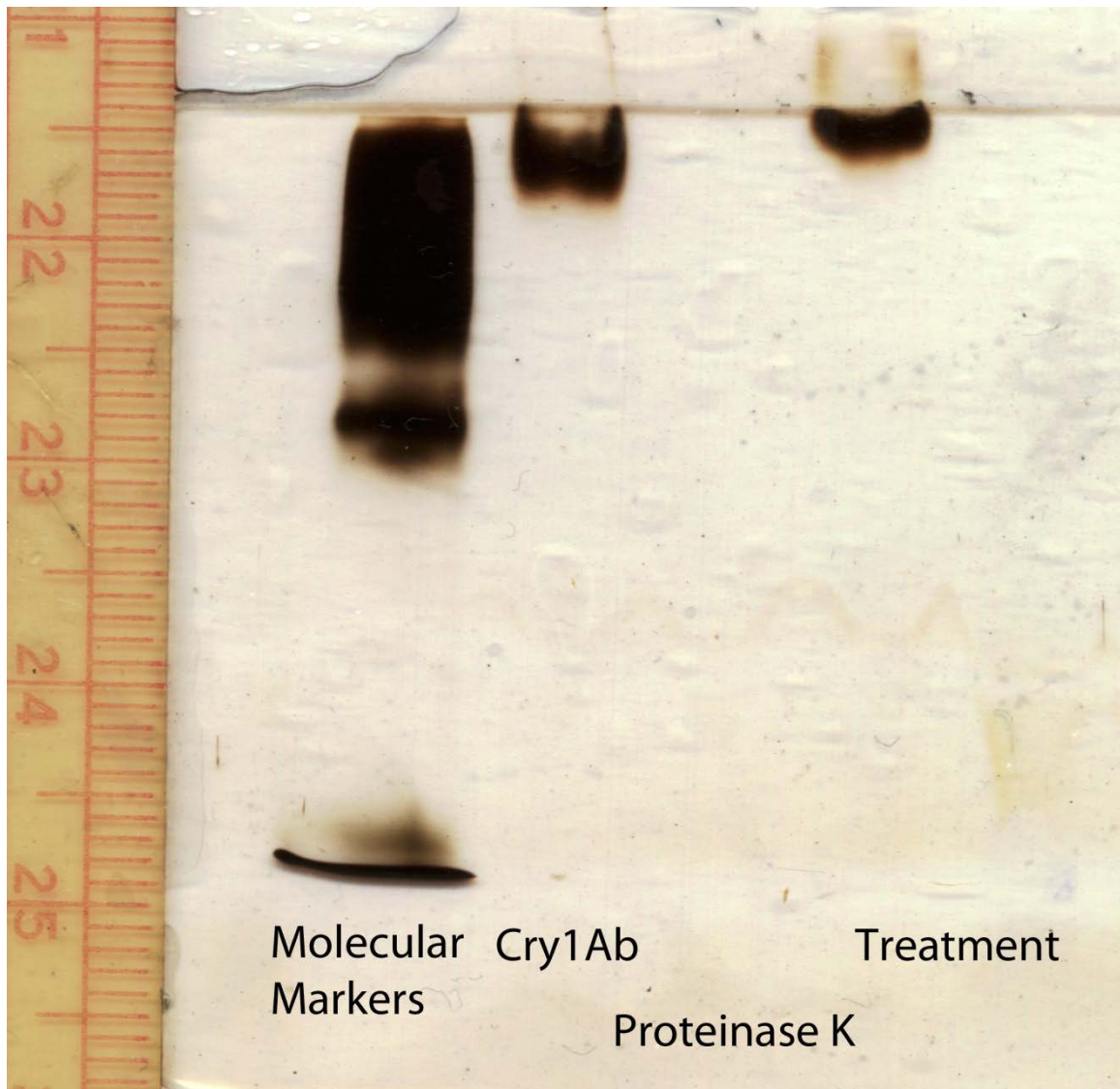


**Figure 2.** Bioassay results from fractions 6-7 of the treatment, Cry1Ab, and proteinase K solutions. Tr = Treatment, Cry1Ab degraded with proteinase K; PrK = Proteinase K enzyme only; Cry = Cry1Ab only. No useful information can be gained from these bioassays, as the solutions were compromised.

### Fraction 12-13 Bioassays



**Figure 3.** Bioassay results from fractions 12-13 of the treatment, Cry1Ab, and proteinase K solutions. Tr = Treatment, Cry1Ab degraded with proteinase K; PrK = Proteinase K enzyme only; Cry = Cry1Ab only. No useful information can be gained from these bioassays, as the solutions were compromised.



**Figure 4.** Results from a native PAGE gel performed on an aliquot of the proteinase K mass digest. Denaturing conditions are not present in native PAGE gels; and proteins present should retain their native conformation. Only one band appears in the digested treatment, suggesting that the protein fragments are associating with each other.

## Tables

**Table 1.** ELISA results of the fractions from the G-50 separatory column experiment. Treatment = Cry1Ab degraded with proteinase K; Pr. K = Proteinase K enzyme only; Cry1Ab = Cry1Ab only; Pure Cry1Ab = protein not fractionated with separatory column. **A** – First ELISA run. Significant amount of protein are still detectable in the treatments that have been degraded by proteinase K. **B** – Second ELISA run. Significantly less protein is present in all samples, indicated the samples were compromised.

	<b>A</b>		<b>B</b>	
Fraction	Absorbance	Concentration	Absorbance	Concentration
Treatment				
4	0.299	1.59	0.088	0.00
5	0.330	2.00	0.352	2.52
6	0.493	4.19	0.111	0.01
7	0.232	0.68	0.029	0.00
12	0.000	0.00	0.014	0.00
13	0.006	0.00	0.012	0.00
Pr. K				
4	0.008	0.00	0.002	0.00
5	-0.004	0.00	0.010	0.00
6	-0.004	0.00	0.011	0.00
7	-0.005	0.00	0.225	1.20
12	-0.005	0.00	0.009	0.00
13	0.019	0.00	0.010	0.00
Cry1Ab				
4	0.007	0.00	0.011	0.00
5	0.354	2.33	0.099	0.00
6	0.840	8.87	0.198	0.92
7	0.917	9.92	0.074	0.00
12	0.050	0.00	0.009	0.00
13	0.028	0.00	0.005	0.00
Pure Cry1Ab	N/A	N/A	0.585	4.95

**Table 2.** Recipes for separating and stacking gel used in native PAGE gel electrophoresis.

<b>Separating Gel</b>	<b>Stacking Gel</b>
1.3 mL 30% acrylamide	0.335 mL 30% acrylamide
3.65 mL 0.375 Tris-HCl, pH 9.4	2.138 mL 0.375 Tris-HCl, pH 8.8
5 $\mu$ L N,N,N',N'-tetramethylethylenediamine	2.5 $\mu$ L N,N,N',N'-tetramethylethylenediamine
50 $\mu$ L 10% (w/v) ammonium persulfate	25 $\mu$ L 10% (w/v) ammonium persulfate

## ACKNOWLEDGEMENT

I would like to thank Jennifer Anderson, Courtney Davis-Vogel, and Anita Unger with DuPont-Pioneer for providing me with training on the ELISA procedure and validation methods. In addition, I would like to thank Chad Boeckman and Kris Sturtz, also with DuPont-Pioneer for providing hands-on training with the bioassay procedure. Keith Bidne with the USDA–ARS Corn Insects and Crop Genetics Research Unit graciously provided me with all the European corn borers I could ever need. Joel Nott, ISU Protein Facility and Brad Coates, USDA–ARS, receive my thanks for helping work through the numerous technical issues encountered during protein analysis. I also would like to thank my fellow ENT-TOX laboratory members for their help and support. I am forever grateful to my co-major professors, Joel Coats and Rick Hellmich for their guidance and support for the past 3.5 years. I am grateful to have had the unconditional love and support of my family throughout my entire graduate career. Finally, none of this would have been possible without the love, support, understanding, and patience of my beautiful wife, Toshia. This one is for you, Anna.